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Odd ways with neutron bombs

That President Ronald Reagan has taken the decision on neutron bombs from which his predecessor shrank in 1979 is no great surprise. But the National Security Council's advice last week — with Secretary of State Alexander Haig apparently dissenting — will reverberate for several months to come. Although the Administration's plan is that the neutron weapons, when manufactured, should be stockpiled in the United States and not shipped to Europe, several democratic European governments will find that circumstance of very little help in dealing with their own electors. The only potential battlefield in which neutron weapons might have a place is in Central Europe but there they could be a powerful reinforcement of the tactical nuclear weapons, euphemistically called "theatre weapons", now or soon to be deployed. It is understandable that Mr Reagan should resist the notion that European governments should hold some kind of veto over the munitions policy of the United States, but it is not merely insensitive of him but dangerous to the cohesion of the chief alliance in which the United States is engaged, to behave as if European opinions are irrelevant.

Scepticism about the feasibility and effectiveness of neutron weapons has by now been largely dissipated. If it is possible to make a high-yield thermonuclear weapon by using a fission bomb to detonate a fusion mixture in such a way that the neutrons produced cause fast fission in a casing made of, say, uranium-238, why not play the same trick within a casing of beryllium, so multiplying the yield of neutrons? The only wonder is that something like this can be done within the diameter of eight-inch howitzer shells. And given the penetrating power of energetic neutrons, there is probably something in the claim that neutron weapons would be relatively more damaging than fission weapons to people rather than property — which is not to say that tanks or other battlefield equipment would remain in working order, ready to be turned against their original owners once the dead (and dying) bodies had been removed. So why is there so much opposition in Europe to what seems to be a sensible improvement of military technology?

Part of the reaction against neutron weapons springs from the widespread conviction that, because all nuclear weapons are abominations, the addition of a new design to existing armouries must be a calamity. The argument is mistaken. European defence is for the time being based on the assumption that nuclear weapons would have to be used in a serious conflict, even if the consequence might be the widespread destruction of European life and property. Neutron weapons, less damaging than "ordinary" nuclear weapons and more easily directed against military personnel, should therefore be more acceptable to European opinion. That is the simple counter-argument. It does not fully meet the subtle European complaint that the availability of relatively usable nuclear weapons will encourage their earlier use in any future European conflict. But that argument cuts both ways. The earlier use of comparatively safe nuclear weapons might just as well serve to prevent a more damaging exchange of nuclear weapons at a later stage as to invite early nuclear retaliation. In the bizarre logic of the nuclear battlefield, in which strategic nuclear weapons are intended to stay forever in wonderland, neutron weapons are a blessing and not a curse.

Unfortunately, especially for President Reagan and for those politicians in Western Europe who must now learn to live with last week's decision, this is not the end of the argument. Protests against the manufacture of neutron weapons may be inconsistent with the assumptions on which the defence of Western Europe is

based, but this does not mean that they count for nothing — especially at the European ballot box. For the past three years, since Chancellor Helmut Schmidt courageously put his political head on the block by saying that he would allow neutron weapons in West Germany (without foreseeing that President Carter would find it politic to stab him in the back), it has been plain that the wayward trend of the past few years in European opinion will be countered only by a resumption of negotiations between the United States and the Soviet Union on nuclear armaments. These negotiations are, in any case, necessary for other and more compelling reasons, the need in Western Europe to modify strategic stand-off by flexibility in East and West European relations chief among them. In the past few weeks, the United States Secretary of State, followed with a needless show of diffidence by the Administration of which he is a part, has been talking hopefully of a resumption of the negotiations on the Salt II treaty (still in limbo) later in the year. (The diffidence is needless because the Administration has no choice.) Has this brave prospect now disappeared?

The most cynical reading of last week's decision on neutron weapons is that it is not (as *Pravda* and the Soviet news agency TASS have been saying) a proof that the United States is not serious about negotiations on strategic weapons but, rather, that it is the opposite — a preparation for Mr Haig's promised talks. For what could be more astute than to stake a claim to the right to deploy neutron weapons in Europe so as to increase the range of possible concessions during a negotiation? The trouble is that last week's meeting of the National Security Council — perhaps the most fully reported on record — seems to have been more iconoclast than that. And even if the underlying calculation is machiavellian, it is wrong. While the Netherlands (reluctantly a potential host for United States cruise missiles and Pershing II missiles in 1983 or thereabouts) still lacks a government, with the danger that the democratic and electoral rot might spread (Belgium?) and with the West German defence budget rising less quickly than expected, the United States Administration may find that it cannot hold the ring in Western Europe, even until Mr Haig's target date later in the year, if it fails to reckon with its allies' ballot boxes, not just with its own. That is a penalty of being a superpower, as the Soviet Union has found in Poland.

Stanford's patent prize

Is Stanford University just lucky? Or unlucky? Or do the problems stemming from its exploitation of the Boyer-Cohen patent, which goes to the root of many present attempts to make a commercial success of genetic manipulation, derive simply from its good sense, over several decades, in recruiting faculty members with skill, imagination and flair? The answer is yes on all three counts.

When universities of all kinds are under serious financial pressures, one that finds itself blessed with what may be a substantial source of revenue in the 1980s (until the patent runs out) will be the envy of both private and public universities in the United States and elsewhere. Even so, it is unlikely that Stanford would have sought the invidious position of being the first university in the United States to have patented a scientific discovery that is already of outstanding importance in research laboratories (where patent protection entails no restriction) and may yet be the basis of a new industry.



Stanford's patent has been made possible by the changes of policy nearly a decade ago at the National Institutes of Health, which made it possible for universities to apply for their own patents even when financial support had come from federal funds. (One irony in the case of the Boyer-Cohen patent is that the University of California never succeeded in negotiating what is called an Institutional Patent Agreement, whence its partnership with Stanford in this venture.) Last year, the doctrine that recipients of federal research grants may nevertheless patent discoveries that arise, and not leave them in the public domain, was extended by the new patent legislation to most agencies of the federal government. The arrangement has some virtues. It provides universities with an independent source of income, and there is a chance that some universities will be more efficient merchants of innovation than the government has been. Given its traditions, Stanford could hardly have stood aside from these developments. In that sense, its bad luck has been thrust upon it.

The university and its members have in the circumstances conducted themselves with the utmost propriety. First, the authors of the discovery have assigned all their personal rights to their respective universities, and will make no financial gain from whatever happens in the next few years. Second, Stanford has apparently consulted widely with the companies likely to be the first in the field with practical applications of the new techniques. Third, patent protection has apparently not been sought outside the United States (but less liberal regulations on prior publication elsewhere might have been an obstacle). Fourth, the terms to would-be licensees are modest, as these things go; neither university seems out to make a quick buck, or an unseemly number of bucks. Even the imminence of the deadline (15 December) before the terms are made stiffer is forgivable, given the advance consultation there has been. And licensees will have to comply with National Institutes of Health guidelines. Finally, Stanford stands out among universities in the United States in having formulated an explicit policy for the seemingly division of the spoils from patent exploitation between the university, the inventors and the departments to which they belong (see *Nature* 18 June, p.524); the Stanford faculty will be debating these proposals early in the new academic year.

So why should there be a fuss, if fuss there is? Most probably the commercial companies, especially those which Stanford has consulted in advance, will take out licences promptly and without complaining that they are being held to ransom. They will know that it will cost them more to buy exclusive (as distinct from non-exclusive) licences for particular applications of genetic manipulation from the genetic engineering companies that have mushroomed in the past few years. Even so, a legal challenge to the patent is more than possible if less than probable, either on the grounds that Boyer and Cohen were not the only authors of the research or that, in the early 1970s, the techniques now patented were not as novel as they may have seemed. Stanford seems prepared for such a challenge (see page 573) and may well be right in thinking that it would win its case. What will concern its friends, those who admire and owe an intellectual debt to Boyer and Cohen as well as those concerned for the reputation of scientific scholarship, is that a wrangle about scientific priority in the courts would be damaging to institutions and people.

Another difficulty goes to the root of current policy in the United States on the exploitation of federally sponsored inventions. There is now a danger that, in the years ahead, drafts of papers for publication will be read with an eye not merely for scientific merit but for exploitability. Moreover, Stanford and the University of California at San Francisco apart, it remains to be seen whether the federal government's new liberality on patents will turn out to be a golden goose or merely another addition to the cost of university administration. A more serious problem is that most universities, within and outside the United States, still lack policies for deciding how the commercial benefits of invention should be divided internally among the interested parties; one obstacle is that the financial interests of the faculty are in many cases already too deeply entrenched. The danger now is that the commercialization of academic work will be reinforced

— and that the benefits will be spread throughout the university system more or less at random, according to the chance that some discoveries will be patentable and others not.

Although in the short run some universities may benefit, all must know that precarious income from patents is no basis for running a university system. Worse, they must also fear that the further they go along the money-making path, the harder it will be for all of them to stick to their proper tasks of teaching and scholarship. And while it may be pleasing to be able to demonstrate to governments that universities can turn their hands to business when they are pushed, that proposition conceals a trap. For the universities have always contributed to socially and commercially valuable innovations. If they now accept the notion that they can do so effectively only when they are rewarded, their place in public esteem will be diminished.

Polytechnics in passing

Once upon a time, when there seemed no limit to the growing demand for higher education in the United Kingdom, the late Mr Antony Crosland, in his capacity of Secretary of State for Education and Science, wove a marvellous spell. In a speech at Woolwich fifteen years ago, Mr Crosland brought into being an entirely novel system of higher education, which he called the "binary" system. One half consisted of the traditional universities. The spearhead of the other half was to be 26 technical colleges, which were redesignated as "polytechnics", urged to provide degree courses for their students (which many already did) but to concentrate on practical things such as engineering, technology, business — the "world of work" as it has since been called. In a curious way, Mr Crosland's magic wand marked the beginning of the universities' fall from grace. If the "other" half was to be the provider of practical higher education, how could the universities fail to be thought effete? But now it is the polytechnics' turn to take a tumble.

After two years of blustering, the British government has now issued a discussion document that will spread gloom and despondency in the polytechnics — and among the local authorities which are their titular sponsors (*Higher education in England outside the universities: policy, funding and management*; Department of Education and Science). The government's supporters, if they have the time to read such a specialized document, may also take fright. For the government now seeks to set up some kind of central body to supervise working of "higher education outside the universities". The local authorities know they have no ground on which to stand — the cheques they write each month to pay the salaries of teachers in polytechnics are immediately discounted by the central government. Worse still, there is a suspicion that the local authorities which happen to be the channels of the central government's cash flow towards the polytechnics (now grown in number to 29) would put higher education high among their priorities. There are cheaper ways of catching votes and, in any case, local authorities are probably as disappointed as the rest of the United Kingdom that the performance of the polytechnics has been so disappointing. Those which have chosen not to ape the universities have all too trendily destroyed their claims to intellectual respectability. There are some exceptions.

The government's solution, modestly called "Model B" in the discussion document, is that the polytechnics (and the rest of non-university higher education) should be controlled by a body analogous to the University Grants Committee. The local authorities have lamely countered with what is called "Model A" — a scheme in which their representatives will call the shots. Everybody knows how the consultation will be resolved. The government's supporters (if they had the time) would be horrified to learn that the crucial argument is the case (spelled out) that central planning of higher education is not merely necessary but possible. For some time yet, it will be forgotten that the most immediate need is to bring the two halves of the binary system into some kind of coordination.

Stanford ready to fight for patent

No challenge as yet in sight

Washington

Officials at Stanford University's Office of Technology Licensing are bracing themselves to meet possible challenges to the patent on the set of basic genetic engineering techniques, used to replicate foreign genes in microorganisms, which was offered for licensing last week.

The patent was awarded last December covering the results of research by Dr Stanley Cohen, of Stanford Medical School's departments of medicine and genetics, and Dr Herbert Boyer of the University of California at San Francisco. Stanford is confident that it can successfully resist any challenge, if necessary in court; but patent attorneys in several large companies are already poring closely over the scientific literature in case they decide to question the basis on which the patent was awarded.

The key question is whether these companies are prepared to accept the terms of the Stanford licence. The university is asking for an initial non-exclusive licence fee of \$10,000 and an annual fee of the same amount for the use of the techniques in research. (University scientists will be exempt.) Royalties on product sales will vary from 1 per cent for the first \$5 million, to 0.5 per cent for sales over \$10 million.

Before deciding on these terms, which are relatively low for patent agreements although these usually offer exclusive licensing rights, the university had consulted several companies — including Eli Lilly, Upjohn, Smith-Kline, Schering-Pough, Du Pont and Monsanto — to gauge their reaction to early drafts.

Several have already indicated that they are prepared to accept Stanford's terms. Dr Peter Farley, for example, president of the Berkeley-based Cetus Corporation, said last week he thought the terms were "very reasonable" and that he expected his company to accept them. Similarly Genentech, of which Dr Boyer is a co-founder, has also indicated that it is likely to agree to the terms.

Other companies, however, are refusing to commit themselves — at least in public — until they have studied the implications of the licensing agreement more closely. Patent attorneys for several large pharmaceutical companies said last week that it was too soon to comment on the actions they may take.

Privately there are rumours that at least one company may decide to challenge the patent in court. But company officials list a

number of factors they will have to take into account.

One is the public impact of a major court case that could rekindle debate on the ethics and safety of genetic engineering. There is also uncertainty over how much sympathy there would be for a company which appeared to be preventing the two universities profiting from discoveries made in their laboratories. (Both Drs Cohen and Boyer have assigned any personal interest in the royalties to their support teaching and research.)

Against this there is the argument put forward by the president of one small company that, since royalty payments will push up product costs — even if only marginally — the taxpayer will be paying twice for the same research.

The principal determinant of whether a challenge is filed, however, is likely to be the cost. Each company must calculate whether the amount of money it could save by not paying Stanford the licence fee and royalties would exceed the amount required to challenge the patent successfully.

According to patent attorneys, any such challenge could take one of two principal forms. A company (or group of companies) could seek a court ruling that the patent had been improperly granted. Or — perhaps more likely — a company could continue to use the recombinant DNA techniques without a licence from

Stanford, waiting for the university to sue for the infringement of patent rights.

In either case there are two likely ways in which the Patent Office's decision to issue the patent might be challenged. The first would be to claim that the research results described in the patent were not "novel" since they were already being used by other scientists, and should therefore be considered part of the so-called "prior art". Alternatively, they could argue that the applicants had failed to show that the particular combination of techniques which they describe for the replication and expression of exogenous genes in microorganisms met the legal criterion of being "non-obvious".

Having granted the patent, the patent examiner responsible must have been convinced that the process described met both of these criteria. However, such a judgement is based largely on the material presented to the Patent Office to support the claim, which was filed by the two universities on behalf of Drs Cohen and Boyer.

A challenge on these grounds would not be a surprise. But officials at Stanford are confident that, although many scientists were involved in the discoveries and developments that laid the basic groundwork, they can demonstrate that scientific collaboration between Cohen and Boyer was sufficiently crucial to justify the claims to the patent.

David Dickson

Antecedents of Cohen-Boyer patent

The text of the Boyer-Cohen patent is a nice bibliography of the early history of genetic manipulation between 1972 and 1974. The patent, granted on 2 December 1980, was filed on 4 January 1980 and partly replaces three previous patent applications filed in 1974, 1976 and 1978.

The abstract of the patent says that "method and composition are provided for the replication of exogenous genes in microorganisms", and refers to the use of either plasmid or virus DNA as replicating elements into which foreign genes can be deliberately inserted for later replication and expression. "The method provides a convenient and efficient way of introducing genetic capability into microorganisms for the production of nucleic acids and proteins, such as medically or commercially important enzymes", and the abstract goes on to list possible applications in the production of drugs, the fixation of nitrogen, fermentation, the "utilisation of specific feedstocks, and the like".

The patent says that the process now protected includes the three steps in the preparation of recombinant plasmids, their use for the transformation of bacteria and the replication and transcription of the recombinant plasmids in transformed

bacteria. In its description of the "prior art" the patent lists a series of papers in *Proc. natn. Acad. Sci. U.S.A.* including those by Cohen *et al.* (69, 2110; 1972), Cohen *et al.* (70, 1293; 1973), Cohen *et al.* (70, 3240; 1973); Cohen *et al.* (71, 1030; 1974), Morrow *et al.* (71, 1743; 1974) and Jackson *et al.* (69, 2904; 1972). In addition, the patent cites the paper by Novick in *Bact. Rev.* (33, 210; 1969).

On the preparation of recombinant plasmids, the patent points to the need for preserving the site of replication and to the convenience of some "phenotypal property" that will aid the selection of transformants. Bacteria are mentioned simply as the most convenient of unicellular organisms with which to work, while usable plasmids are said to range in molecular weight from 1 to 50 million daltons. The plasmid pSC101 (developed in 1972 by Cohen *et al.*) is mentioned as a convenient plasmid for cloning purposes, while the techniques of cleaving plasmid DNA and its subsequent ligation to other pieces of DNA are also described.

Acknowledging that some recombinant plasmids can arise naturally, the patent says that the techniques covered make possible the construction of artificial recombinants, including those between

bacteria "which do not exchange information naturally" and between bacteria and eukaryotic organisms "which cannot naturally occur".

The patent says that the techniques can be used with genes to form other bacterial cells, mammalian cells, plant cells and so on. The cloning of multiple genes is mentioned and the production of multiple copies of peptide hormones said to be of especial importance in the cases of parathyroid hormone, growth hormone, gonadotropin, insulin, ACTH, somatostatin and prolactin. Other useful products of genetic manipulation listed

include serum proteins, ferritin, myoglobin, interferin (*sic*), kinins and transcortin.

While illustrating the claims made with specific examples, the patent says that "it is obvious that certain changes and modifications may be practised" within its scope. Among the specific claims are for the ligation both of blunt-ended and "staggered" DNA molecules, to form recombinant plasmids.

On the face of things, the patent would seem not to cover the use of vehicles other than plasmids for the cloning of DNA, so that techniques of genetic manipulation involving the use of animal virus grown on susceptible cells in tissue culture would seem not to be protected. Nor is the development of cloning vectors able to switch between eukaryotic and prokaryotic cells. Yeasts are, however, mentioned.

The patent makes no claim to protection for organisms or plasmids with particular properties, presumably because it was not known whether organisms could be patented until the Supreme Court decided earlier this year in favour of a patent application on behalf of Dr A.M. Chakrabarty by the General Electric Company for a strain of *Pseudomonas* designed for digesting oil spills. Stanford is apparently waiting to hear whether its applications for protection for specific bacterial cloning strains will be successful.

Soviet biotechnology

Further resolve

Soviet progress in biotechnology is being hampered by shortages of reagents and apparatus, and delays in the construction of new facilities. Experiments are limited in scale, and too little is being done to implement theoretical results.

This is the tenor of last month's resolution of the Central Committee of the Party and the Council of Ministers on the further development of biotechnology. The official Soviet commitment to biotechnology began suddenly in May 1974 with a similar resolution, which in effect gave biotechnology priority status and funding from then rather than from the start of the succeeding five-year plan.

Reviewing progress in the past seven years, the Central Committee and Council of Ministers last month noted that the 1974 resolution had introduced "radical changes" in the biological sciences, especially in molecular biology, bio-organic chemistry, molecular genetics and immunology. Research cadres had been trained within a very short time, and in several fields Soviet scientists had attained a "leading position in world science". Despite high hopes, a flourishing biotechnology industry had not materialized.

The resolution suggests, however, that this is not simply because of the difficulty of turning research results into practice. The resolution charges the Academy of

Sciences, the State Committee for Science and Technology, the State Planning Committee (Gosplan) and the governments of the union republics with the task of ensuring the conditions for these results to be introduced in agriculture, medicine and industry. But it also calls for intensified fundamental research both directly within the framework of the Academy of Sciences, and the ministries.

The State Committee for Science and Technology, the Academy of Sciences and Gosplan are to adopt a special target-orientated research programme in biotechnology, which will be coordinated by the State Committee's special council on biotechnology and the academy.

The supply problem remains. The resolution merely says that means of ensuring supplies of equipment, reagents, "biochemical preparations", computer technology and an information base in biology and biotechnology have been "noted", but makes no suggestion of what form these measures are to take. **Vera Rich**

US air pollution

Reagan retreats

Washington

Unexpectedly strong opposition has forced the Reagan Administration to retreat from plans to seek sweeping changes in US air pollution legislation. Initially Mr Reagan had announced his intention to demand a substantial weakening of the Clean Air Act when it comes up for renewal by Congress next month; but following objections not only from environmentalist groups and Democratic congressmen, but also from several leading members of the Republican-dominated Senate, the Administration said last week that it was looking for more modest amendments.

One of the most significant shifts in the Administration's position has been its decision to continue to use health standards and "sound scientific data", rather than economic costs, as the basic principle for determining how air pollution should be regulated. Mr Reagan's budget director, Mr David Stockman, as well as members of his Council of Economic Advisors and outside industrial groups, had strongly urged that cost-benefit techniques be added to the statutory requirements of the Clean Air Act, first passed by Congress in 1970.

However, Mrs Anne M. Gorsuch, the new administrator of the Environmental Agency, announced last week that the Administration did not intend to go far down this particular path. Listing the basic principles to be followed in developing legislation to extend the act, she said that "cost-benefit analysis should not be included as statutory criteria in setting these standards", but that standards "should be based on sound scientific data demonstrating where air quality represents

Europe waits to see

European pharmaceutical companies have reacted cautiously to the announcement by Stanford University that licences on the Boyer-Cohen patent are now for sale. But many of them are huddled with their patent lawyers.

Under the terms of US patent law, companies outside the United States will be obliged to apply for licences only if they intend to market within the United States products made with techniques covered by the patent. Because most European companies are anxious not to foreclose future markets, however, they face the same dilemma as US companies in deciding whether to pay the licence fee and subsequent royalties.

Most companies this week were unwilling to guess what they may in the end decide to do, although it seems that some of them have already been consulted (through their American subsidiaries) by Stanford. Some point out that Stanford's advertised terms are reasonable, but also say that the university has been well advised in fixing a low fee and royalty rate for a non-exclusive licence to exploit a patent as general as Boyer-Cohen. Larger proprietary costs will accrue, they say, when the time comes to buy an exclusive licence to use a more specialized technique from one of the companies set up in the past few years to carry out research and development in genetic engineering.

Some legal anomalies stand out. Will, for example, a European manufacturer that seeks to market in the United States a product made by a process developed by a non-American genetic engineering company itself be required to pay a royalty, or will the legal obligation fall on the company that carried out the research and development?

In the circumstances, three options are being considered: to ignore the licence and face Stanford when products are placed on the US market; to challenge the patent; or simply to pay up. European companies, however, are unlikely to initiate direct challenges, preferring to wait and see what happens in the United States.

Judy Redfearn

real health risks".

Both supporters and critics of the Administration agree that the concept of "real health risks" will now become the central focus of debate over new regulations — and that the decision may reflect recent rulings by the US Supreme Court which, while denying that cost-benefit calculations need be used in setting standards, have also emphasized the legal importance of scientific or epidemiological evidence of existing levels of risks.

Several of the principals announced by Mrs Gorsuch have pleased industry. For example, emission restrictions for nitrous oxides on new cars will be eased. Under present legislation, all cars produced from this year onwards are allowed to produce only one gramme of nitrous oxide for every mile travelled. The Environmental Protection Agency intends to raise this limit to two grammes, the level at which it stood between 1977 and 1980 and a move which it is claimed could save \$100 on the price of a family car.

There will also be a relaxation in a particularly controversial section of the present legislation aimed at preventing the "significant deterioration" of air quality, even in areas where present levels of pollution are relatively low. This provision stems from a legal interpretation of the 1970 Act following court cases brought up by several large environmental groups; it has been bitterly contested by industry on the grounds that it restricts the growth of new industries outside existing urban areas.

Mrs Gorsuch said last week that the present programme for the prevention of significant air quality deterioration "should be maintained for the protection of park and wilderness areas". She said that protection in other areas should be based on uniform technology requirements for pollution control.

The Environmental Protection Agency has also committed itself to asking for more funds from Congress for acid rain research. This has recently become a major source of conflict between the United States and Canada, which has accused Washington of failing to comply with an agreement signed last August to reduce air pollution from automobiles and large coal-burning facilities. The Administration, with the utilities industry, is now arguing that considerably more research is required before the need for clear action is demonstrated, a position which is unlikely to be warmly greeted in Ottawa.

Critics of the Administration's earlier proposals, contained in draft legislation leaked to the press by congressional Democrats in May, have been given other concessions. Senator Robert T. Stafford of Vermont, for example, insisted that no change should be made to the principle that the young, the elderly and other high-risk groups should be given an extra margin of protection from the effects of polluted air. Furthermore, pollution standards will continue to be set at the federal level and

not, as industrial groups hoping for a more sympathetic hearing had asked, by individual states.

The Reagan Administration's decision to limit its proposed changes seems largely based on a realization that, unlike the cuts in public spending and taxation, there is no national consensus on the need for a major retreat on environmental protection. White House officials are said to have been concerned about the impact of recent aggressive anti-environmentalist statements by Interior Secretary James Watt, and have asked him to adopt a more conciliatory attitude.

But if the battle over the Clean Air Act extension is not going to be as fierce as once promised, it will still be heated. The National Clean Air Coalition claim that even the Administration's more limited proposals are a "blueprint for destruction" of the present laws. Mrs Gorsuch says that, even with the suggested changes, the quality of the nation's air will improve, but "at a more reasoned pace". Congressional hearings will begin next month.

David Dickson

Science jobs famine

Bangalore

Nearly three million scientists in India are unemployed and an equal number are working in posts requiring much lower qualifications, for lower pay than a government clerk receives. These were among the worrying facts which came to light at a recent meeting of the Science Advisory Committee of the Union Cabinet, headed by Planning Commission member and agricultural scientist Dr M. S. Swaminathan, and held to discuss ways of making the best use of the country's science and technology personnel, particularly those unemployed.

For instance, more than 50 per cent of scientists and engineers employed at the prestigious Bhabha Atomic Research Centre draw a monthly salary of less than \$75. And nearly 25 per cent of the personnel working in government-sponsored research and development institutions have a monthly pay packet of less than \$120. However, people working in science and technology in the private sector are better off, seven per cent of them earning \$200 a month.

According to a note circulated to members of the committee, unless the annual rate of industrial growth rises to four per cent there is little prospect of speeding up the absorption of unemployed science and technology personnel into jobs.

The note further recommends regulation of intake of such personnel for training, more informal education, facilities for improving qualifications while on the job and greater interaction between industrial and academic scientists.

B. Radhakrishna Rao

Industrial noise

Let them hear

Britain's Health and Safety Commission is tackling the difficult business of regulating noise at work. Last week it published draft regulations in a consultative document intended to pave the way for legislation which will place a statutory obligation on employers to protect workers from noise. A voluntary code of practice, drawn up in 1972, is being widely ignored, according to the commission.

Devising effective legislation is, however, proving controversial — noise regulations are notoriously difficult to enforce and statutory levels of noise are fiercely disputed. The consultative document proposes limits of no more than 90 decibels(A) — a weighted decibel unit discounting inaudible frequencies — averaged over an eight-hour day and no more than 600 pascals of peak sound pressure. But the trades unions, in particular, argue that as hearing damage can occur at lower levels, the daily average limit should be set initially at 85 dB(A) and later lowered to 80 dB(A).

The number of British workers exposed to high noise levels is considerable. The commission estimates that 50 per cent of production workers (about 2.5 million people) are exposed to more than 80 dB(A) and 10 per cent (500,000 people) to more than 90 dB(A). Nobody seems to have disputed the effect of noise on hearing since the late 1960s. Using the work of Burns and Robinson, the commission estimates that roughly 32 per cent of people exposed to 100 dB(A) over a working lifetime will suffer a 50 dB hearing loss by the age of 65.

The commission has settled for the 90 dB(A) limit on the basis that the hearing of 21 per cent of workers previously exposed to more than 100 dB(A) will be protected. Reducing the limit to 80 dB(A) would save the hearing of only a further 8 per cent. Clearly acknowledging the problems of reducing noise, it argues that a 90 dB(A) limit will give greater benefit for cost.

Under the commission's proposals, however, employers would not be freed from obligations even at lower noise levels. The draft regulations require that they reduce any noise likely to damage hearing to the "lowest level reasonably practicable", which they would have to work out for themselves. Employers fear that this general but unspecific obligation may expose them to civil suits from all those who suffer from hearing loss.

The draft regulations require that at noise levels above 90 dB(A) employers must survey noise, train staff, keep exposure records and appoint a qualified noise adviser. Workers exposed to 105 dB(A) would have to receive regular hearing checks. The proposed regulations also oblige employees to make full use of noise control measures and manufacturers to reduce noise levels of industrial

machinery to below 84 dB(A) if possible. The commission has no estimate of the cost of its proposal.

On this occasion, Britain's Health and Safety Commission seems to be a step ahead of the European Commission, whose directive is still some way off. But the signs are that Europe will go for a limit of 85 dB(A). **Judy Redfearn**

Bulgarian astrophysics

Cosmic celebrations

The "Interkosmos-Bulgaria-1300" satellite, Bulgaria's cosmic celebration of 1,300 years of statehood, was launched last Friday (7 August) to the surprise of the Bulgarian public, who had expected it would form the climax of the celebrations in October.

The designation of the satellite was equally unexpected — although the original plan was for a single celebratory satellite, analogous to the Polish Interkosmos-Kopernik-500 in 1972, it was announced earlier this year that there would be two satellites, one containing Bulgarian experiments only and the other a mixed Soviet and Bulgarian payload. However, only in the Moscow radio coverage of last Friday's launch was it made clear that the mixed payload was already in orbit — aboard the Priroda-Meteor satellite launched on 10 July.

In the Bulgarian celebrations, the timing has proved unfortunate, since the head of the jubilee committee, Lyudmila Zhivkova (the daughter of First Secretary Todor Zhivkov) died suddenly on 20 July.

Scientifically, however, the satellite is operating well, and has done much to offset Bulgarian disappointment that the Interkosmos manned programme did not allow them a second chance of a cosmonaut aboard a Salyut craft. By way of consolation, Georgi Ivanov, whose Soyuz transporter failed to achieve a link-up with Salyut, was invited to attend the launch and spoke warmly of Soviet-Bulgarian cooperation.

Although the experiments on the satellite (a modified "Meteor" meteorological probe) are said to have been produced "with the assistance of Soviet scientists", the twelve experiments comprising the 350-kg payload are of Bulgarian design, and concentrate on those branches of space physics of particular interest to the Bulgarian space team, including plasma, high energy charged particle studies, atmospheric luminescence and magnetosphere studies. According to Dr Kiril Serafimov, chairman of the Bulgarian Space Research Council, a special study will be made of ionosphere troughs and equatorial anomalies in the magnetosphere, which it is hoped will provide valuable data for such applied fields as radio-wave propagation, the mechanism of rare meteorological anomalies and the radiation balance of the Earth. **Vera Rich**

Carcinogenicity testing

Well, yes and no

Washington

Semantic juggling has allowed US health officials to escape the embarrassment of discovering two reports on the widely-used chemical dimethyl terephthalate (DMT) which are virtually identical but contain directly conflicting conclusions about its carcinogenicity.

Re-examination of the test data has raised a dilemma for scientists with the Department of Health and Human Services' National Toxicology Program (NTP) that is frequently faced by regulatory agencies: how to present policy-makers with the results of ambiguous animal tests.

The solution proposed by NTP's peer review committee is to describe data on increased cancer rates in male mice as being "statistically significant" but "biologically equivocal".

DMT is widely used to provide flexibility in plastic products from food wrapping to bottles. The source of the confusion is a technical report on its potential carcinogenicity prepared in the 1970s for the now-defunct Clearinghouse on Environmental Carcinogens of the National Cancer Institute (NCI).

The tests were carried out by a private contractor, Hazleton Laboratories. They revealed no evidence of increased cancer rates among male and female rats, or among female mice exposed to low or high doses of DMT. However, a 27 per cent incidence of lung tumours was found among male mice receiving the high doses, considerably higher than the 4 per cent incidence in a group of matched controls. On the basis of these data, which were approved by an NCI peer review group, a report was published describing DMT as a carcinogen in male mice.

Soon afterwards, however, the report was challenged by scientists working with Hercules Incorporated, a major manufacturer of DMT. They claimed that the relatively low incidence of lung tumours among the matched controls was out of line with results from other control groups. NCI scientists reassessed the Hazleton data, this time using for comparison not the matched control group, but pooled results from three other control groups associated with different experiments which had spent overlapping periods of time in the same room. The latter groups revealed a lung tumour incidence of between 10 and 18 per cent over a two-year period, much closer to the 27 per cent of the exposed group.

A revised version of the technical report was subsequently issued through the National Technical Information Service (this time without peer review) which stated in the summary that the new data indicated that DMT was not a carcinogen.

Several research libraries, however, still

have copies of the first version on their shelves. And the apparent discrepancy between the two reports was brought to the attention of NTP, which has taken on the responsibilities of the NCI clearinghouse, three months ago.

Re-examination of the data revealed that the tests appeared to have been properly conducted, even though the incidence of tumours in the matched controls seemed inordinately low. "We view the incidences of total lung tumours in the matched male controls with some suspicion" reported Dr John Moore, deputy director of NTP.

A report on the NTP staff review was brought before the peer review panel of the programme's board of scientific counsellors last month. Discussion soon focused on whether, in revising the original report, the NCI staff had been correct in ignoring the matched control data and

One more journal

A new scientific journal is to be launched at the beginning of 1982 by the European Molecular Biology Organisation (EMBO). One of the objectives of *The EMBO Journal* is to redress the balance between Europe and the United States where, it is argued, the predominant and preeminent position won by American journals in the publication of molecular biology has put European molecular biologists at a disadvantage.

The new journal, which has been in the air for the past three years, was finally agreed after a ballot of the 500 members of the organization held in January. The journal will resemble *Proceedings of the National Academy of Sciences of the United States of America* in that it will publish papers communicated by any of its members, each of whom will be rationed to a maximum of five papers a year by non-members of the organization.

A statement by the publishers of the new journal, IRL Press Limited of Oxford, England, says that EMBO members will be expected to take responsibility for the "scientific standard" of the papers they communicate. One of the obvious difficulties will stem from the heterogeneity of the membership, which is elected, and the differing interpretation of what molecular biology consists of in various European countries.

The editor of *The EMBO Journal* will be Dr John Tooze, executive secretary of EMBO and also, at present, the editor of *Trends in Biochemical Sciences*. One feature of the new journal is said to be speed of publication — the publishers have undertaken to print manuscripts accepted by the editor within twelve weeks of their receipt. A pilot issue of the journal is to be made available in September.

basing their reassessment on the three other control groups; or whether all four groups should have been pooled into a single set of controls.

Various members of the committee expressed unease with the statistician's technique of ignoring so-called "outsider" data points which did not appear consistent with the other data being evaluated — but which, according to the biologists, there might be no particularly good scientific reason to disregard.

Members of the NTP peer review committee agreed to recommend the aggregate data as the basis of the evaluation about carcinogenicity. But it left the problem of deciding how to describe evidence that was in Dr Moore's words, "something between equivocal and positive".

The proposed solution was an appendix to the second version of the technical report, which will give details of the new analysis and point out that, statistically, the raised incidence of lung tumours in mice exposed to DMT is "highly significant". But the appendix will add that the results are also biologically equivocal — in other words the data do not provide clear evidence on whether DMT is a carcinogen.

The next step rests with the Food and Drug Administration's Bureau of Foods. Previously the bureau's cancer assessment committee had decided, on the strength of the NCI report, that no action was needed against DMT. With the new interpretation from NTP, the committee will have to reassess its position.

Meanwhile the NTP's board of scientific counsellors is meeting soon to discuss whether a more rigorous system of classification, possibly based on procedures developed by the International Agency for Research on Cancer in France, should be adopted in the United States. The DMT episode will encourage their efforts in this direction.

David Dickson

Alternative energy

Enter the Nibe

Brussels

By 1995, about 15 per cent of Denmark's electricity requirements will be met by wind power — if the Danish Ministry of Environment goes through with its plan to plant 3,000 windmills across the country. The plan is being seriously considered and has now been passed to the relevant regional and local authorities for their views.

Denmark is more dependent on imported energy than most other industrialized countries, having to provide 95 per cent of primary energy from outside sources. The only domestic form of primary energy is natural gas under the North Sea, and this will not start flowing until 1984. So research into renewable energy sources has been given high priority.

Even before the last government plan, a 1979 law provided government subsidies of up to 30 per cent of the cost of small

domestic windmills. These have to be of an approved type, and the electricity utility companies are prepared to buy any surplus electricity from the owner.

The new plan is altogether more ambitious. The 630 kW three-blade rotor mills, called Nibe wind turbines, were developed under the 1977–80 wind energy research programme which cost Dkr 39 million (\$7.2 million). The Nibe windmills are raised on a 41-metre concrete tower and the span across the turbine blades is about 40 metres. The mills will operate in wind speeds of between 21 and 90 kilometres per hour. The research programme has evidently ironed out most of the major technical problems, leaving the economic and environmental issues to be debated.

Denmark is a small, densely populated country, so the choice of suitable sites is severely limited. The windmills cannot be placed in areas where they would interfere with recreational, agricultural or other economic interests. If it were possible to find coastal sites for all 3,000 mills, electricity production would be 25 per cent greater than if they were all inland.

Somewhat surprisingly, there is evidence to show a relationship between electricity demand and high winds in Denmark. Apparently heating requirements are greater the stronger the wind blows and, conveniently, this would be countered by more power from the windmills.

But before the windmill ideas take off, it will be necessary to bring down the unit cost of construction — or the other sources of electricity will have become prohibitively expensive. Present cost of wind-generated electricity is \$2,000 per kW of installed power.

As with nuclear power stations, however, there may be objections from local residents. Anyone living near one of these Nibe wind turbines would have to put up with noise, interference with radio and television, and landscape eyesores. But Danish public opinion is likely to favour wind power and Denmark seems likely to be the first country to launch a major commercial wind power programme.

Jasper Becker

India in space

Apple limps on

New Delhi

Although still operating on power from only one of its two solar panels, India's "Apple" experimental telecommunications satellite has now been successfully placed into geostationary orbit 36,000 kilometres above Sumatra in Indonesia. Apple's solid-fuelled apogee boost motor was fired on 22 June to achieve near-geosynchronous orbit but one of the solar panels failed to respond to radio commands and did not open. Despite this the satellite was stabilized and two other major manoeuvres were performed to correct an eastward drift and increase the orbital height. On 16 July Apple reached its intended orbit, with its antenna pointing towards Nagpur in central India.

The experiments in telecommunications techniques began on 22 July, and the bulk of the programme should be completed in spite of the reduction in the satellite's useful life from two years to one and the 160 watts of power available instead of the planned 280 watts. After the earlier anxiety, scientists at Apple Mission Control Centre in Sriharikota are buoyant at having manoeuvred the spacecraft successfully.

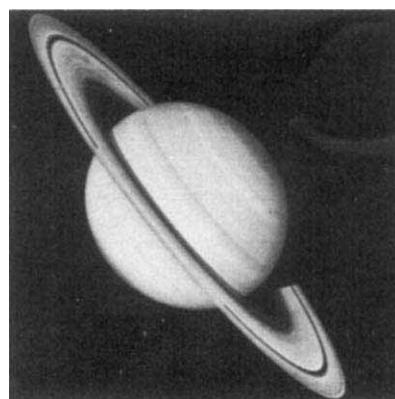
Good news was long overdue for the Indian space programme, for only 9 days after its launch aboard India's own SLV-3 launcher on 31 May, the remote sensing satellite Rohini II had burned up in the atmosphere — far short of its expected lifetime of 90 days. Rohini I was also ill-fated, crashing into the Bay of Bengal in August 1980 only a few days after launch.

Meanwhile, provided it is still functioning, Apple is to be used to broadcast nationwide an address from Prime Minister Indira Gandhi on Independence Day, 15 August. Only a few metropolitan centres will be able to pick up signals, but it marks a beginning to the plan to bring communications up to date throughout the Indian subcontinent.

Sunil Saraf

Saturn seen by Voyager 2 as the spacecraft approaches its encounter with the planet on 26 August, en route to Uranus (1986) and Neptune (1989). In the light of the results of the Voyager 1 encounter last November, the experimental programme of Voyager 2 has been considerably revised. Next week's *Nature* is a special Saturn issue containing new results from Voyager 1 and a description of the Voyager 2 mission by Dr Edward Stone, the Voyager mission's director.

The issue will also contain the usual features, including a report on the total synthesis of an interferon gene.



CORRESPONDENCE

Scientists in the Soviet Union

SIR — News of oppression crosses my desk daily. It is so commonplace that after a while its impact is almost lost. Like physicians who must become hardened to deal daily with death, human rights workers become somewhat inured to a daily fare of suffering. Nothing breaks down this shell more quickly than a visit to the victims of oppression.

As executive director of the Committee of Concerned Scientists, I went to the Soviet Union recently to investigate at first hand the trying circumstances confronting *refusnik* and dissident scientists. I found that the familiar litany of persecutions continues unabated. Most scientists who apply for emigration or speak out in defence of human rights are dismissed from their jobs. The regime then does its utmost to ensure their intellectual death by isolating them from their colleagues. In their personal lives too these individuals are plagued by countless harassments. Their homes are searched, their telephones disconnected, their mail intercepted. Scientists who apply to emigrate are routinely refused exit visas on spurious grounds. Anti-semitism is rife in all phases of academic life from admission to universities, to conferral of degrees, to obtaining employment. Those who have fallen from grace are frequently threatened with prosecution on criminal charges. Some are tried and sentenced to prison, labour camp and internal exile. These violations of human rights and scientific freedom are not new developments. Regrettably we have been hearing about them for some time.

I had learned much about this bleak situation during my four years with the Committee of Concerned Scientists. But I was not prepared for reports of the latest acts of persecution. Harassment has assumed new forms. Even prisoners are subjected to additional vengeful punishment. The health and very lives of prisoners of conscience Sergei Kovalev, Yuri Orlov and Anatoly Shcharansky are in grave jeopardy. Lengthy periods of solitary confinement, reduced rations and inadequate medical attention have taken their toll.

Other ominous trends in the treatment of *refusniks* and dissidents emerged from my discussions with leaders of these communities. First and most distressing is the wave of trials of scientists. Never before have so many activist scientists been threatened with lengthy sentences at one time. In Kiev Vladimir Kislik was sentenced to three years in a labour camp. Several years ago authorities had sought to imprison him for "compromising secrets" in an article published in an international journal. Yet this research had been officially cleared for publication some five years earlier. When confronted with a flood of protests from Kislik's Western colleagues pointing to the absurdity of this charge, the authorities backed off. In June, however, they achieved their goal through a contrived criminal charge. Computer scientist Viktor Brailovsky, a leader of the Moscow Sunday Scientific Seminar since its inception nine years ago, has been sentenced to five years of internal exile for advocating the right to emigrate, deemed defamatory to the Soviet state. In Leningrad,

refusnik mathematician Yevgeny Lein will also soon face trial. Interestingly, Kislik, Brailovsky and Lein all played key roles in organizing seminars designed to maintain the scientific skills of *refusnik* scientists.

The attempt by the Soviet authorities to close down these unofficial seminars is another cause for alarm. While seminars in provincial cities, such as Kiev and Vilnius, were squelched, those in Moscow and Leningrad had operated over a period of years with relatively little interference until recently. The Moscow Sunday Seminar, which had been blocked in the wake of Viktor Brailovsky's arrest in November, was able to resume regular Sunday sessions unmolested in early February. But this respite was short-lived; the authorities have shut it down three times since the end of April. On one Sunday during my visit at the end of May, the KGB barred the way to three different apartments making it impossible for a session to take place. Also in Moscow Alexander Lerner's seminar on mathematical biology, which recently held its 200th session, was blocked twice in April and May. In mid-May the seminars went into official recess for the summer. Our Soviet colleagues anxiously await the official reaction when they attempt to resume their

meetings in the fall.

Still another cause for concern is the use of academics as an instrument for persecution. Many scientists are now refused exit permits not because of allegations (usually unfounded) that they know state secrets, but simply because they are well educated. In fact the head of the administration department of the Communist Party's Central Committee bluntly told scientists that they are being detained "because you have degrees". At the same time the authorities are punishing scientists by abrogating their advanced degrees. Indeed three *refusnik* scientists told me their degrees had been revoked for "unpatriotic behaviour," that is, exercising their legal right in applying to emigrate.

While the situation of *refusnik* and dissident scientists has deteriorated alarmingly over the past year, this course is reversible. We can have an impact. Witness the return of Benjamin Levich, Mark Azbel, Valentin Turchin and others to productive scientific life due to the untiring efforts of Western scholars. We cannot afford to become hardened to the plight of oppressed colleagues who have much to contribute to a vital international science.

DOROTHY HIRSCH

*Executive Director,
Committee of Concerned Scientists,
New York, USA*

An Irish question

SIR — In your editorial of 25 June 1981 (p.601), you quite rightly in my view criticized the Science and Engineering Research Council (SERC) for turning down the proposal "that there should in future be an exchange of graduate students between the the United Kingdom and the Irish Republic on the modest scale of a dozen or so a year". You may not be aware that the Department of Education (N. Ireland) awards postgraduate studentships to good graduates who have normally been resident in N. Ireland for at least 3 years immediately preceding the start of the proposed period of study. One type of research studentship, the so-called XNI award, is tenable at institutions in Great Britain or the Republic of Ireland or exceptionally, outside the British Isles. This arrangement, which I believe has existed for many years, meets the points you make about student exchange in respect of graduates from N. Ireland. Unfortunately, there is no reciprocal arrangement in the Irish Republic.

I have had several enquiries from final-year honours students reading biochemistry in the Republic who would like to have carried out postgraduate research in this department. The only suggestion I have been able to make is that the prospective research students should apply to Queen's University for a Visiting Studentship of which there are about two per annum for the whole world including Great Britain. Competition is very keen for these studentships and the prospects of success are small. Perhaps you should give the Ministry of Education in the Irish Republic a nudge in addition to the black mark which you awarded to SERC.

*Queen's University,
Belfast, UK*

D.T. ELMORE

University cuts

SIR — Union members have expressed particular concern over two aspects of the leading article "Change wanted" (*Nature* 11 June, p.442).

(1) It seems to accept the principle of cutting back on funding of universities, an extraordinary position for a journal concerned with science and research.

(2) It does not answer the questions and propositions posed by Swinnerton-Dyer but suggests the necessary financial savings can be made by college managements acting in a managerial capacity and shedding staffs from all sections except academic.

With regard to this last point, there is no suggestion as to how teaching laboratories and research groups could maintain their standards or volume of work when numbers of technicians and other ancillary staff, already at a bare minimum in many institutes, are reduced even further.

You cannot fail to be aware that the whole question of Swinnerton-Dyer is currently the subject of intense discussion not only in the university but also between the University of London and the trade unions that represent all sections of staff. We feel that these discussions should continue in a serious way on the basis of the evidence submitted in the discussion documents and that they should not be prejudiced by statements such as yours that the "armies of ancillary staff" should be chopped by the administrative action of the management of the schools.

M. OSMUNDSON
C. SCOTT
L. WALDOCK

*Queen Elizabeth College branches
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London, UK*

NEWS AND VIEWS

The location of nucleosomes in chromatin: specific or statistical?

from Roger Kornberg

THE finding that histones are arrayed as nucleosomes along a DNA molecule immediately raised the question of whether the array occurs in a specific relationship, or 'phase', with respect to the DNA sequence¹. Were this the case, then regulatory molecules might act in a novel way. For example, a protein responsible for setting a specific phase would determine which DNA sequences are protected by nucleosomes and which sequences are exposed in the regions between them. An alteration in this protein could shift the array of nucleosomes, exposing one set of sequences and covering up another. A single protein could thus control the function of a series of regulatory signals on DNA from a distance.

This intriguing possibility was investigated by comparing the distribution of nucleosomes on rat liver chromatin containing single-copy DNA with the distribution of nucleosomes formed essentially at random by reconstitution of DNA from the same source². The two distributions proved to be indistinguishable: no greater specificity with respect to sequence was apparent in the location of nucleosomes on the rat liver chromatin than in their location on the reconstituted chromatin. This ruled out a specific phase relationship of nucleosomes with rat liver DNA sequences.

An important assumption behind the notion of nucleosome phasing was that the spacing of nucleosomes was constant along a chromatin fibre and an array of nucleosomes thus periodic, akin to a linear crystal. If, however, there were no regular spacing, no long-range order, then a specific phase relationship between nucleosomes and DNA sequences could not persist for any distance along the DNA. This assumption was tested by two separate experiments which established that the spacing of nucleosomes is not constant, but variable within a cell^{2,3}.

Nonetheless, there have been several reports indicating that even if nucleosome spacing is irregular, it is not random; and

some indicating that it is both regular and non-random. Most of these experiments depend on the use of micrococcal nuclease, which cleaves DNA preferentially between nucleosomes, and they fall into three groups according to the extent of digestion. In studies of genes for heat-shock proteins and histones in *Drosophila melanogaster*^{4,5}, very brief digestion of chromatin was followed by extraction of the DNA, cleavage with a restriction enzyme, gel electrophoresis and Southern hybridization with a restriction fragment produced by the same enzyme. This procedure reveals the length of DNA from the restriction site to the various points of cleavage in chromatin by micrococcal nuclease. Well defined locations of nucleosomes will result in a discrete set of DNA lengths and thus a pattern of bands, whereas random locations are expected to give a continuous distribution. Invariably a series of bands extending for thousands of base pairs was observed. The bands were irregularly spaced and those approximately 130–230 base pairs (bp) apart were attributed to defined locations of nucleosomes, although this attribution is a little problematic because the nucleosome core DNA is generally not less than 146 bp long.

In experiments with genes for tRNA in chicken embryos⁶, micrococcal nuclease digestion was more extensive, sufficient to generate a mixture of nucleosome monomers, dimers, trimers, and so on. The mixture was fractionated according to numbers of nucleosomes by sedimentation in a sucrose gradient, and the location of tRNA genes within individual oligomers determined by extraction of the DNA and cleavage with restriction enzymes, as well as by other procedures. Once again, the locations of nucleosomes with respect to DNA sequence were found to be non-random, though only for a distance of about five nucleosomes from the tRNA gene after which they became indistinct. In

this case, the defined locations were regularly spaced, with a periodicity the same as that found by micrococcal nuclease digestion of bulk chicken embryo chromatin.

In a third set of experiments, very extensive digestion of chromatin with micrococcal nuclease was used to degrade all the DNA to 146-bp core fragments, which were then mapped with respect to specific sequences. A unique location of nucleosomes in African green monkey satellite chromatin⁷ and a set of alternative locations of nucleosomes on genes for 5S rRNA in *Xenopus laevis*⁸ were found in this way.

Additional evidence of both random^{9–16} and non-random^{17–19} locations of nucleosomes has come from restriction enzyme digestion of chromatin and from other approaches. We are thus left with a serious conflict that needs to be resolved. One view, widely shared²⁰, is that 'phasing' of nucleosomes is a general phenomenon of considerable biological interest, despite any evidence to the contrary. An alternative view is that the two sets of apparently conflicting data may be reconciled, for example by the following simple picture.

Nucleosomes are deposited essentially at random on DNA, subject to two constraints. First, they are not formed on DNA associated with other proteins, for example sequence-specific regulatory molecules such as repressors and activators, but are restricted to the regions of DNA between such molecules. Second, nucleosomes occupy 166 bp of DNA^{21–23} and they may not overlap one another. The average locations of nucleosomes subject to these constraints have been determined by computation²⁴. It is only average properties that are of interest, because all experiments are done on millions of genomes. Remarkably, although nucleosomes are deposited at random, the computations show a distribution with a periodicity and degree of order similar to that revealed by micrococcal nuclease digestion of bulk chromatin. Furthermore, near the

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boundary of a region of nucleosomes, specific locations are strongly favoured, with regular spacings identical to the periodicity of bulk chromatin and with probabilities that gradually diminish until there is less than a 10 per cent preference over neighbouring locations at a distance beyond about five nucleosomes from the boundary.

The simple statistical picture can account for the evidence for non-random locations of nucleosomes on chicken tRNA genes, provided that there is a sequence-specific protein or structure in the vicinity of these genes to form a boundary for a region of nucleosomes. The presence of regulatory proteins near the 5' end of the genes would satisfy this requirement. The similarity between the distribution of nucleosomes computed on statistical grounds and that found in the experiments on tRNA genes is striking. Both distributions show a regular spacing of nucleosomes identical to the periodicity of bulk chromatin, and both show defined locations over a range of about five nucleosomes but not far beyond. Whereas these two distributions appear so very alike, both differ markedly from that reported for heat-shock and histone genes in *Drosophila*, where a pattern of irregularly spaced bands extending for thousands of base pairs was produced by very brief digestion with micrococcal nuclease and interpreted in terms of specific locations of nucleosomes. This interpretation can be questioned on several grounds, the most serious of which concerns the preference of micrococcal nuclease for cleaving certain DNA sequences²⁵. It has recently been shown that striking patterns of bands are produced by digestion of naked DNA and furthermore, that the patterns resulting from digestion of naked DNA and chromatin are the same^{16,26-29}. This specificity of digestion poses the greatest problem when the period of digestion is very brief. It creates a need for additional evidence to support the assignment of a band pattern to an array of nucleosomes, such as the physical demonstration of nucleosomes by sedimentation in a sucrose gradient.

A further difficulty is that the interpretation in terms of an irregular spacing but well defined location of nucleosomes leads to a contradiction. Irregular spacing can only be explained by sequence-specific binding of histones to DNA. This implies that the location of nucleosomes is the same in all tissues of an organism since the DNA sequence remains the same. However, the spacing of nucleosomes has been shown^{30,31} to vary between different tissues, so the locations of the nucleosomes cannot remain constant but must vary as well.

Although the formation of nucleosomes is unlikely to be strongly sequence specific, neither is it likely to be completely independent of sequence, and the

assumption of random deposition of nucleosomes in the statistical picture can only be regarded as an approximation. Other proteins that associate with nucleic acids in a uniform way, such as the coat protein of tobacco mosaic virus, show some preference, perhaps by an order of magnitude or more, for binding to some sequences over others. It would be surprising if a similar preference were not shown by histones. Indeed, there is clear evidence for such sequence preference in the distribution of nucleosomes on polyoma DNA³², and the non-random locations of nucleosomes on satellite DNA and 5S rRNA genes mentioned above may be explained in this way (or by this effect combined with that of proteins bound at specific sites, which will be frequent in these DNA repeat regions).

Perhaps the most striking example of specificity in the location of nucleosomes so far discovered is the specific absence of nucleosomes from a region of about 400 bp encompassing the replication origin and

promoters of the SV40 chromosome. The DNA in this region appears naked, as judged from accessibility to restriction enzymes and electron microscopy³³⁻³⁷. The 'DNase I hypersensitive site' adjacent to the 5' end of many genes undergoing transcription^{4,38-40} may represent a similar region of naked DNA. The exclusion of nucleosomes from these regions is most simply explained by the sorts of constraint on the location of nucleosomes mentioned above. Thus the binding of non-histone proteins to any two sequences less than 166 bp apart will prevent the formation of a nucleosome on the entire region between them. T antigen is known to bind in the vicinity of the replication origin of SV40, and other regulatory proteins are doubtless bound near the 5' end of active genes as well.

In all the cases of non-random locations of nucleosomes discussed here, the key question concerning biological significance is the degree of preference over adjacent locations. All known regulatory proteins bind their cognate DNA sequences with affinity many orders of magnitude higher than unrelated DNA. The processes that would be regulated by mechanisms involving nucleosomes surely require at least as great precision. The degree of specificity that can arise in the statistical picture described above is at most an order of magnitude and so would seem unlikely to have any biological role. It remains to be determined whether any greater precision is achieved in other examples of non-random locations of nucleosomes. Unfortunately the methods in use are incapable of giving this information. The experiments are carried out, for the most part, using enzymes that cleave anywhere in the region between nucleosomes, and the data that result are mostly qualitative, in the form of bands in gels, which are visually striking even when intensity of only a factor of two separates them from the background.

The biological significance of specific locations of nucleosomes, if they are found, is apparent, but the advantages of a statistical distribution of nucleosomes, while not as obvious, are also great. A statistical distribution allows the packaging of DNA without regard to the length or the sequence involved. Were there genuine 'phasing' of nucleosomes, the loss or addition of a single base pair could have deleterious effects. A statistical distribution, on the other hand, would accommodate the extraordinary variations of genomes observed in nature.

Thus there is no convincing evidence for ordered arrays of nucleosomes in a fixed relation to the DNA sequence over a distance of many thousands of base pairs. Constraints on a random distribution of nucleosomes resulting from the presence of other proteins at specific DNA sites may impose some short-range order, but the degree of this order is unlikely to be sufficient for biological specificity. []

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Stripped for action

from Peter D. Chantler

STRIPPING proteins down in order to assess the individual functions of their peptide subunits has long been a preoccupation of biochemists. Hexameric myosin molecules are an ideal choice for this practice and an investigation of the role of the essential light chain in vertebrate skeletal muscle myosin was recently reported in *Nature* by Wagner and Giniger (Vol. 292, p.560).

All muscle myosin molecules contain two heavy chains (molecular weight $\sim 2 \times 10^5$) and two pairs of low-molecular-weight light chains (molecular weight $\sim 2 \times 10^4$ each). One of these pairs comprises the DTNB light chains, so called because they were first partially dissociated from rabbit skeletal myosin using DTNB (5,5'-dithiobis [2-nitrobenzoic acid]). However, because their removal (in amounts greater than 1.5 moles per mole myosin) was found to have no effect on the $(Ca^{2+} + K^+ - EDTA)$ - or actin-activated ATPase activities of myosin, they have also been described as 'non-essential'. In contrast, the other pair of light chains became known as 'essential' because their removal by various treatments always seemed to result in an irreversible loss of these ATPase activities; although as heavy chain denaturation appeared to precede essential light chain removal, the precise role of the essential light chain remained ambiguous.

The problem is further complicated in skeletal muscle by the presence of myosin isozymes. Thus there are two forms of essential light chain, A1 and A2; both exist within a single muscle cell¹ and the presence of myosin homodimers and heterodimers of A1 and A2 light chains has been confirmed²⁻⁴. In addition, at least two forms of heavy chain exist within fast skeletal myosin⁵.

Using the results of earlier workers as a guide, Wagner and Weeds⁶ found that in 4.7 M NH_4Cl , 2 mM EDTA and 2 mM dithiothreitol, pH 7.0, an equilibrium existed between dissociated essential light chain and heavy chain⁶. A 10-min incubation at 4°C did not adversely affect the ATPase activities. Exchange experiments were easily feasible in the presence of excess light chain, using isolated chymotryptic subfragments of myosin, S-1 (A1) and S-1 (A2), containing A1 and A2 essential light chains respectively. These two types of myosin 'head' are easily separated on a DEAE-cellulose column and lack the non-essential DTNB light chain, which is digested away by the chymotrypsin leaving the essential light chain intact. The results of such exchange experiments, now well known,

were that the kinetic parameters of the two subfragments appeared to be determined by the type of essential light chain, provided the assays were performed at low ionic strength (6mM)⁶; at higher ionic strength the parameters are virtually indistinguishable for the two isozymes⁷.

To study the separate roles of the heavy chain and essential light chain, Wagner and Giniger have now combined two powerful approaches: the above exchange procedure and the antibody column techniques of Holt and Lowey², with the additional trick of including nucleotide in all these procedures to stabilize the nude heavy-chain fragment (so simple that one wonders why nucleotides were not included in dissociation experiments years ago). Thus, S-1 (A2) was incubated for 5 min at 4°C in 4.7 M NH_4Cl , 5 mM ATP, 2 mM EDTA, 2 mM dithiothreitol, 0.1 M imidazole, pH 7.0, and then applied to an anti-essential light-chain antibody column equilibrated in the same buffer. The antibody is fully operational in these conditions and removes the dissociated A2 light chain. The subfragment eluting from the column still possesses about 10 per cent of A2 light chains but retains 50-100 per cent of the ATPase activities and 100 per cent of the actin-binding capabilities of S-1(A2) that has been similarly treated but not passed down the antibody column. Contaminating S-1 (A2) can be removed by second passage of the material through the antibody column in the absence of NH_4Cl , to give a pure, active heavy-chain fragment. These results have been substantiated by Sivaramakrishnan and Burke (submitted for publication) who have used a different approach to produce light chain-free heavy chains of rabbit S1; the heavy chains retain full ATPase and actin-binding capabilities.

Taken together, these results show that the essential light chain is, in fact, inessential! They suggest that the active site and the actin-binding site are exclusive to the heavy chain in the head region of myosin. The essential light chain (now a misnomer) can modulate this activity at low ionic strength but the activity is primarily determined by the heavy chain. The combined work of Wagner and Giniger and of Sivaramakrishnan and Burke represents the first clear demonstration of an active myosin heavy chain, free of light chains (notwithstanding the active minimyosin heavy chain from *Acanthamoeba*⁸ which is regarded as peculiar among myosins).

The corollary of pulling peptides apart is to reconstitute the original protein by adding together the constituents. Wagner and Giniger have not reported such an experiment, although it is possible and will be reported shortly by Sivaramakrishnan



100 years ago

PROF. Raoul Pictet of Geneva, who has been giving his attention of late to marine architecture, announces, according to the *Times* correspondent, a discovery which, if his anticipations be realised, will effect a revolution in the art of shipbuilding and greatly augment the speed of sea-going and other ships. The discovery consists in a new method of construction and such an arrangement of the keel as will diminish the resistance of the water to the lowest possible point. Vessels built in the fashion devised by Prof. Pictet, instead of sinking their bows in the water as their speed increases, will rise out of the water the faster they go, in such a way that the only parts exposed to the friction of the water will be the sides of the hull and the neighbourhood of the wheel. In other words, ships thus constructed, instead of pushing their way through the water, will glide over it. According to the professor's calculations, steamers built after his design will attain a speed of 50 to 60 kilometres the hour. A model steamer on the principle he has discovered is being constructed at Geneva.

From *Nature* 24, 18 August, 362, 1881.

and Burke. In a sense, however, the thunder of such a reconstitution experiment has been stolen by direct exchange experiments producing hybrid myosins⁹ using essential light chains of skeletal myosin and heavy chains of cardiac myosin, and vice versa. The results of these hybrid experiments indicate that the ATPase properties of myosin are largely determined by the heavy chain, in agreement with the results above on S-1.

Thus relatively minor roles have been assigned to the essential light chain and the DTNB light chain of skeletal myosin. Furthermore, the role of phosphorylation of the DTNB light chain in skeletal myosin remains an enigma. This is in contrast with the role of the regulatory light chain in scallop myosin (chemically related to the DTNB light chain), also deduced from stripping experiments, of governing

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actomyosin interactions by determining the structure of the calcium-specific switch situated on the scallop myosin molecule^{10,11}. There is also increasing evidence for involvement of the essential light chain of scallop myosin in this regulatory mechanism^{12,13}. In addition, many recent papers point to the importance of regulatory light chain phosphorylation in smooth muscle and non-muscle myosins.

Is it possible then that the DTNB and

essential light chains of vertebrate skeletal myosin represent vestigial peptides — protein subunits whose principal role of regulation has long since been usurped by thin-filament control and the evolution of an architecture devoted entirely to efficient, repetitive contraction? In this context the increased stability conferred on the heavy chain by the presence of the essential light chain and the small modulation of actin binding by DTNB

light-chain phosphorylation¹⁴ may be seen as evolutionary vestiges of a once important regulatory role; the loss of the calcium-specific site could be seen as an example of 'silent' mutations. Whatever the merits of such a depressing (for some) speculation, it is clear that Wagner and Giniger, together with Sivaramakrishnan and Burke, have performed a long awaited and much needed experiment and thereby given us ample food for thought.

A glimpse of new results: the preliminary Solar Maximum Mission data

from Robert Rosner

THE first extensive reports of results from the (partially defunct) Solar Maximum Mission (SMM) satellite have recently appeared in the *Astrophysical Journal Letters* (244; L133, 1981). Following the precedent set by the Einstein Observatory consortium two years ago, the SMM experimental teams joined in submitting a series of papers which has now been published as a single issue of the Letters. Most teams contributed two papers: the first describing the particular experiment and its in-flight observational capabilities, and summarizing some of the principal preliminary results; the second focusing on a particular event or observation. The SMM observational programme also had a strong ground-based component, which is in evidence both in the accompanying article by Rust *et al.*¹ and in the individual papers (in which simultaneous radio and other ground-based data figure quite strongly in guiding the interpretations). The result is a useful overview of solar physics research conducted from SMM (as well as of the ancillary ground-based work), concentrating on the solar flare problem, and a good indicator of the quality of the data base generated by SMM. Astronomers wishing to acquaint themselves with the current status of the solar flare problem can do little better than to combine the reading of this volume of *Astrophysical Journal Letters* with a perusal of the Skylab Solar Flare monograph². SMM will clearly provide significant observational constraints for flare theorists.

The SMM satellite carried on board seven instruments (see refs 3,4 and refs therein), six of which were spectroscopic, specifically optimized to observe dynamic events in the solar chromosphere and corona; the seventh instrument was a very broad-band (active cavity) radiometer intended primarily for temporal studies of the total solar irradiance. The energy range of the spectrometers spanned from the visible (6,583 Å) to 160 MeV, with the only significant gap in the extreme UV (in which, unfortunately, fall a number of

strong resonance lines from plasma species at transition region temperatures, such as O VI [1,032 Å]). I will attempt here to describe what I believe are the main features of the results reported by the seven experimental teams.

Coronagraph/polarimeter (C/P). The C/P was a coronagraph capable of high-speed multicolour photometry and polarimetry. Built by the High Altitude Observatory (Boulder; L.L. House, Principal Investigator [PI]), it was specifically designed to observe transient coronal mass ejection with high time resolution and short response time (to flare onset alarms) as close to the solar limb as possible³. The instrument had a spatial resolution of 100 arc s and was capable of observations as close as $\sim 0.51 R_{\odot}$ from the solar limb; the spectral range covered was 4,435–6,583 Å, with particular emphasis in these reports on the classic coronal green line of Fe XIV (5,303 Å) and the red line of H α (6,563 Å). The preliminary data presented by Wagner *et al.*⁶ of the large coronal transient of 7 April 1980 are largely morphological, as the detailed calibration required to retrieve plasma diagnostic information had apparently not been completed. The images presented are, however, spectacular; successive images show rapid motions in both radial and non-radial directions, with peak velocities near 650 km s⁻¹ at the outermost edges of the transient. Simultaneous radio observations made with the Culgoora three-frequency radioheliograph established the presence of a moving type IV source very near the outward-moving loop (surprisingly the 80-MHz source moved differently both in time and space from the 43-MHz source). Analysis of the radio data shows that the magnetic energy density in the transient region exceeded the thermal energy density, a conclusion which is essentially independent of the emission mechanism hypothesized as responsible for the observed radiation; and, if the instrument

calibration bears out the estimates for excess mass in the transient made by Wagner *et al.*, one finds that in this event, the kinetic energy density is comparable with the magnetic energy density, and so far exceeds (by at least an order of magnitude) the internal (thermal) energy density. The conclusion — that estimates of total flare energies released based solely on the observed radiative fluxes can be mistaken by up to several orders of magnitude — is a caution well known from previous flare studies⁷. Furthermore, these observations confirm the conclusions of the Skylab Solar Flare workshop that loop transients must be driven largely by Lorentzian forces, rather than by gas-pressure gradients⁸.

Ultraviolet spectrometer and polarimeter (UVSP). The UVSP team (E. Tandberg-Hanssen, PI) comprised members of many collaborating institutions, including the Marshall and Goddard Space Flight Centers, the High Altitude Observatory (Boulder) and the Lockheed Palo Alto Research Laboratory. Spanning the spectral range 1,170–3,600 Å, the telescope/spectrograph laid claim to both the highest spatial (3 × 3 arc s) and spectral ($\lambda/\Delta\lambda \sim 150,000$) resolution on SMM; in addition, the instrument could carry out polarimetric observations, using a MgF₂ waveplate (and thus was, in principle, capable of the first measurements of the four Stokes parameters in the solar UV spectrum below 2,000 Å). One of the most surprising results of SMM in fact derives from the UVSP polarimetry: using observations of circular polarization of the 1,548 Å line of C IV above a large sunspot, Tandberg-Hanssen *et al.*⁹ derive a line-of-sight magnetic field strength of $\sim 1,100 \pm 300$ G. This remarkably high field strength (considering the height of formation of the C IV line) is based on an assumed Doppler width, and so it is crucial that the measurement be confirmed in further detailed studies. If it were confirmed, standard models for the extrapolation of magnetic fields above sunspots might need

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substantial revision. Another observational feature reported by Tandberg-Hanssen *et al.* is the detection of significant oscillatory mass motions above sunspots. Using the C IV line once again, the UVSP found oscillations with periods of ~ 130 and ~ 170 s over two different spots; the fluctuations were in phase over four adjacent pixels (which were repeatedly rastered) but out of phase by ~ 180 deg in the blue and red sides of the line for any given pixel. It is not certain to what process such oscillatory motions are attributable, although calculations of the wave motions of flux tubes by H. Spruit (Max Planck Institute) and B. Roberts (St Andrews University) and collaborators appear very promising. Studies of the spatial and temporal behaviour of the density in the transition region of solar active regions, as determined from the intensity ratio of the Si IV (1,402.8 Å) and O IV (1,401.2 Å) lines, show further evidence for a substantial level of dynamical behaviour in 'quiescent' regions. Thus, the UVSP has observed very large (~ 5 – 10 times) spatial variations in transition region densities on scales of ~ 4 arc s, as well as temporal variations on time scales of minutes or less. From the point of view of modelling, it is relevant to ask whether the observed fluctuations take place within magnetically 'confined' plasma structures (for example, whether they occur at the re-entrant footpoints of hot coronal 'loops'). If so, then these data suggest that the transition regions of coronal 'loops' are far more complex in their behaviour than allowed for in standard models for quiescent loop structures. Turning to yet more dynamic phenomena, Woodgate *et al.*¹⁰ discuss thoroughly the morphological aspects of the limb flares of 30 April 1980, as seen in the C IV line discussed above; several of the other teams have in fact found it useful to take advantage of the high spatial resolution imaging capability of the UVSP to place their (lower-resolution) observations in the proper geometric 'context'. The main conclusion of Woodgate *et al.* seems to be that, although the pre-flare state resembles the scenario of models for flare initiation of, for example, Canfield *et al.*¹¹ and Heyvaerts *et al.*¹² — a small loop of emerging flux rising into an overlying complex of magnetic loops — the detailed evolution of loop brightening and plasma motions does not. This conclusion must be tempered by the realization that it may not be reasonable to confront a theory which is designed to explain the 'primary' flare features with morphological details it may be, in principle, incapable of accounting for (see ref. 2 for a particularly lucid account of this difficulty in flare research).

Soft X-ray polychromator (XRP). The XRP actually consisted of two instruments, the flat crystal spectrometer (FCS), with spatial resolution of 12×12 arc s and spectral resolution of $\sim 1,000$ at 20 Å, and the bent crystal spectrometer

(BCS), with a substantially lower spatial resolution of 6×6 arc min, but far higher spectral resolution ($\lambda/\Delta\lambda \sim 25,000$). The experimental team reflects a collaborative effort between Lockheed Palo Alto (L.W. Acton, PI), Mullard Space Sciences Laboratory (J.L. Culhane, PI) and the Rutherford and Appleton Laboratories (A.H. Gabriel, PI). One of the most interesting results reported of the SMM mission is the XRP observations of strong line broadening in both quiescent active regions and flaring regions. For example, Acton *et al.*¹³ discuss the observations of quiescent active regions with the FCS; the spectra show substantial broadening beyond the thermal line width (which is estimated on the basis of electron temperatures inferred from the measured Ne IX/Mg XI line flux ratio). Assuming an isothermal model, Acton and collaborators derive r.m.s. speeds of $\sim 100 \pm 28$ km s⁻¹ and argue convincingly that the excess broadening is probably due to random motions on spatial scales far smaller than the 'loop' structures themselves. How these observations relate to specific proposed coronal heating mechanisms is not yet clear because virtually all the current contenders predict some level of flow 'turbulence'. Turning to higher spectral resolution, a nice example of the power of spectroscopy is provided by the analysis of BCS flare data by Culhane *et al.*¹⁴. These authors show in a succinct and convincing way that: (1) electron temperatures via the satellite-to-resonance line flux ratio for Ca XIX *k* and Fe XXV *j* lines can be obtained, and the Fe lines are formed in different regions than the Ca lines (suggested by differences in derived temperatures, temperature evolution and intensity light curves; see also previous observations of the Ca XIX and Fe XXV lines by Doschek *et al.*¹⁵ and Feldman *et al.*¹⁶); (2) line widths are strongly variable during the course of a flare, starting with rather broad profiles and narrowing significantly after the hard X-ray impulsive event; the broadening may be associated with turbulent flow speeds of ~ 60 – 100 km s⁻¹; (3) fluorescence, rather than ionization by fast electrons, is the most plausible excitation mechanism for Fe K α emission in at least some cases. In one case, however, the excellent temporal coincidence between a hard X-ray burst and an early Fe K α 'spike' suggests (following calculations) that the energetic electrons responsible for the hard X-ray burst also excite the K α transitions (in fact, calculation of the expected K α flux from fluorescence yields far too little emission in this case).

Hard X-ray imaging spectrometer (HXIS). The HXIS was an imaging spectrometer with a field of view of 2 min 40 s $\times$ 2 min 40 s of arc at a resolution of 8×8 arc s, and a field of view of 6 min 24 s $\times$ 6 min 24 s of arc at a resolution of 32×32 arc s. It had some energy resolution covering the spectral range 3.5–30 keV in six channels and variable time resolution in

the range 0.5–7.0 s. The HXIS team (C. de Jager, PI) represents a collaboration between the Astronomical Institute at Utrecht and the University of Birmingham (UK). Its two contributions focused on providing a complete evolutionary description of the morphology and plasma characteristics of three flares observed in April 1980 (7 and 10 April: Hoyng *et al.*¹⁷; 30 April: van Beek *et al.*¹⁸). Although the gross morphological characteristics of flares seen in X rays were well established by the Skylab observations, these HXIS data illustrate the value of extending time-resolved imaging to higher photon energies. The flare observations in early April showed emission regions characterized by a hard spectrum to be spatially associated with, and to overlie, the brightest H α emission patches, and furthermore that associated, softer emission components tended to lie between the harder components. If the emission source geometry is assumed to be that of a coronal 'loop', then a natural and immediate interpretation of these data is that the hard X rays are emitted at the loop footpoints (where the H α emission would be expected to take place), whereas the softer X rays are emitted at the top portion of a loop. If one views the footpoint excitation as a result of particle (electron) beams impinging on the dense footpoint boundary, then the 'thick target' model for the hard X-ray emission is clearly favoured. Further support for 'streaming' of fast electrons comes from the late-April flare data. Van Beek *et al.* show that the onset of flare emission could be associated with a very compact source and that the subsequent development of an extended 'tongue'-like component, whose temperature is higher than that of the compact component, is then consistent with production of energetic electrons within the compact component and subsequent streaming or diffusion into the larger (but magnetically confined) volume. In particular, this sequence of events is consistent with the time scale for the development of the extended source and the lack of clear evidence for large-scale mass motions within this source, and accounts well for the simultaneous appearance of an H α 'tongue' spatially coincident with the extended X-ray tongue. Note that the above argument depends crucially on the assumed source geometry and that at X-ray wavelengths, SMM observers invoke the Skylab observations to justify their choice of loop geometry.

Hard X-ray burst spectrometer (HXRBS). The HXRBS (built by the Goddard Space Flight Center; K.J. Frost, PI) was not an imaging instrument and so provided no spatial resolution (the field of view encompassed the entire Sun). However, the lack of spatial resolution was compensated by high sensitivity and extremely high (1 ms) temporal resolution (because the probability of more than one flare occurring is low and because the

quiescent corona contributes a negligible fraction of the observed emission, source confusion for the HXRBS is of little concern); the spectrometer covered the ~ 25 –400 keV energy range in 15 channels. The HXRBS team chose to discuss observations of three flares (29 March, 30 April and 7 June 1980) for which the time-resolved data allowed access to a previously inaccessible observational domain (Dennis *et al.*¹⁹; Orwig *et al.*²⁰). The combination of large effective area with high potential time resolution allowed this instrument temporally to resolve several hard X-ray bursts into subfeatures: a combination of short (~ 1 s), quasi-periodic 'sub'-bursts, separated by 1–2 s and characterized by a fairly hard spectrum, and a much softer, slowly varying component. In addition, HXRBS detected persistent intensity fluctuations significant down to the 100 ms level and spectral variability down to a time scale of seconds. By taking advantage of the capability for simultaneous observations with the other SMM (as well as ground-based) instruments, Orwig *et al.* were also able to compare the details of their light curves of the 30 April flare with those obtained with the UVSP, HXIS and BCS. The onset of Ca XIX resonance line broadening discussed above seems to be well correlated in time with the onset of the impulsive burst and essentially all the flux observed by the HXRBS probably derives from a small loop (seen by the UVSP) coincident with one of HXIS's 8×8 arc s pixels.

γ -Ray spectrometer (GRS). The short wavelength limit of solar radiative emission was explored by the GRS, an instrument whose experimental team (E.L. Chupp, PI) reflects a collaboration between the University of New Hampshire, the Max Planck Institute for Physics and Astrophysics (Munich) and the Naval Research Laboratory (Washington, DC). At these energies, the Sun emits both a continuum and a number of γ -ray lines; the latter are thought to arise from the interaction of fast ions with the denser portions of the solar outer atmosphere. γ -Ray emission is thus a direct diagnostic for the presence of, for example, accelerated ions and examination of the spectral and temporal behaviour of the emission provides a clue to the characteristic of the incident fast particles (compare with ref. 21, p.117). The GRS explored the energy range ~ 0.3 –9 MeV with an energy resolution of ~ 7 per cent (FWHM at 662 keV) and a time resolution of ~ 16 s (or, using the 'burst window' 64 ms time resolution in the very limited single-channel energy range 330 ± 25 keV). The two contributions of the GRS team (Chupp *et al.*²²; Ryan *et al.*²³) focused on analysis of continuum and line emission from two flares; only in one were both the continuum and lines detected. Of particular interest are the data for the flare of 7 June 1980, for which the first detailed observations of the full light curve of the 2.223 MeV line from

the reaction $n + p \rightarrow {}^2\text{H} + \gamma$ were obtained. The power of nuclear physics to 'recreate' the sequence of events which must have led to the emission of this γ -ray line (as well as the continuum) is nicely laid out: not only must both electrons and nucleons have been accelerated, but the time constraint on neutron production (imposed by the requirement that the fast neutrons be thermalized before ${}^1\text{H}$ capture and the 2.223 MeV photon emission occur) is such that the fast ions responsible for their production must have been accelerated in virtual time coincidence with the fast electrons. This result imposes a non-trivial constraint on particle acceleration mechanisms anticipated (as an SMM result) at the time of the Skylab Solar Flare workshop (see ref. 21, p.162).

Active cavity radiometer irradiance monitor (ACRIM). ACRIM was the only SMM instrument not specifically designed for the study of flare transients. The culmination of an evolutionary sequence of successively more refined radiometers built by R.C. Willson (Jet Propulsion Laboratory), ACRIM was the first instrument put into orbit which had some assurance of resolving solar irradiance fluctuations to better than 0.1 per cent, and thus promised to aid in resolving the raging debate on the constancy of the solar irradiance. This promise has evidently been fulfilled. Two distinct questions arise: first, is there any evidence for variation in the solar constant (such as integrating the solar bolometric flux at some fixed radius over 4π steradians)? Second, is there any evidence for variability in the solar irradiance (such as in the total flux as seen from ~ 1 AU)? The second question (which is the only one a single spacecraft can

sensibly answer) is intimately connected with a classic problem of solar physics: where does the radiative energy 'missing' from sunspots go? Several alternative solutions have been proposed, all constrained by the long known fact that there is (to some photometric precision) no evidence for a bright ring surrounding sunspots (compare with refs 24–26 and refs therein). These alternatives include redistribution of the missing energy over a sufficiently large portion of the solar surface, redistribution in spectral range (to the far UV or X rays), redistribution in transmission mode (conversion of radiative flux to magnetohydrodynamic waves), redistribution in time (storage in the convection zone, followed by gradual release over a substantial portion of the surface) and, most recently, spatial and angular redistribution (by means of the solar faculae²⁷). The first observational problem is of course to detect the effect of the passage of a sunspot across the solar disk on the total solar irradiance. This goal has been admirably met by ACRIM: Willson and Hudson²⁸ demonstrate fluctuations of the total solar irradiance of the 0.05 per cent level; but a correlation with specific solar surface activity could not be established in this preliminary analysis. The vagaries of publication are such that even before the appearance of the 'SMM' *Astrophysical Journal Letters*, Willson and his collaborators published²⁹ a more extensive analysis of their observations in *Science*; thus it is already well known that the 'dips' in total irradiance seen by ACRIM correlate well with the passage of sunspots across the solar disk (confirming previous Nimbus data suggesting, but not establishing, a similar correlation). The next task will be to identify definitively the source of the 'peaks' in total irradiance; it should then be possible to answer the 'missing flux' problem of sunspots.

The preliminary results discussed above suggest that, as the SMM data are more fully analysed and their implications for theoretical work better appreciated, SMM will contribute significantly to solar research, particularly in the areas of spatially and/or temporally resolved spectroscopy, imaging at hard X-ray photon energies and solar irradiance variability. It is to be expected that the interpretation of SMM data, which understandably received relatively less weight in these presentations of preliminary results, will play a far greater part in the next round of SMM presentations. It is noteworthy that the new Japanese satellite, ASTRO-A, is observing solar atmospheric dynamics with instruments very roughly comparable with the BCS and HXRBS. Given the premature failure of SMM's pointing capability, it is fortunate that it may, after all, be possible to follow up some of the discoveries revealed by the studies of the SMM data set.

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ARTICLES

Jupiter tail phenomena upstream from Saturn

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Voyager 2 plasma wave and plasma probe measurements from February 1981 suggest that phenomena associated with a well defined tail of Jupiter have been detected at a distance of about 6,200 R_J . This indicates that Saturn's magnetosphere will be affected by the jovian tail and that by comparing Voyager 1 and 2 observations information on the physics of Saturn's magnetosphere can be obtained.

BEFORE Voyager arrived at Jupiter, the possibility of encounters with Jupiter's extended tail during the subsequent Jupiter-to-Saturn phase was noted¹. It was shown that in the spring and summer of 1981, Voyager 2 and Saturn would both cross the expected region of tail or wake about 7,000–8,000 R_J downstream from Jupiter and that this tail might cause a change in characteristics of Saturn's magnetosphere². In August 1980, Voyager investigators found evidence that Jupiter's tail extends out to at least 700 R_J (ref. 3). Here, we report Voyager 2 plasma wave measurements from February 1981 which are evidence for a well-defined distant tail of Jupiter at least 3 AU downstream, indicating that Saturn's magnetosphere will be affected by the jovian tail^{1,2,4}.

Tail encounter at $R = 6,200 R_J$

Jovian continuum radiation was detected by the Voyager 1 plasma wave instrument as soon as the spacecraft entered the dayside magnetosphere⁵, but the most intense signals were observed when Voyagers 1 and 2 were both traversing the nightside tail lobes^{6–8}. Indeed, we regard the detection of this continuum radiation from the Voyager 16-channel spectrum analysers as the single plasma wave measurement characteristic most clearly associated with a spacecraft location within Jupiter's magnetosphere. Therefore, when Voyager 1 and 2 detected roughly similar plasma wave emissions after leaving the nominal close-in magnetosphere, simultaneous measurements from the on-board plasma probes were used³ to identify whether the spacecraft had re-entered Jupiter's magnetosphere. One Voyager 1 event (with $R \approx 163 R_J$, $\phi \approx 245^\circ$) and six Voyager 2 events (with $184 R_J \leq R \leq 706 R_J$, and $\phi \approx 224$ – 225°) were recognized as true encounters with the extended tail (here, ϕ is the azimuth angle in a right-handed Jupiter-centred system, with $\phi = 0$ oriented towards the Sun). The other 19 events, detected when the Voyagers were still in the solar wind, were interpreted in terms of leakage from an extended tail into a low-density solar wind trough, which would act as a wave guide, allowing signals from the distant tail to reach the spacecraft.

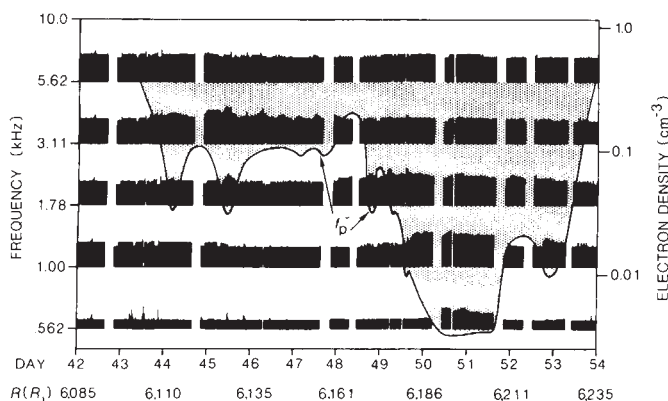


Fig. 1 Time profiles of average electric field strengths detected in five spectrum-analyser channels of the Voyager 2 plasma wave instrument. The scale on the right-hand side refers to the plasma density that gives an equivalent electron plasma frequency ($f_p = 9,000\sqrt{N_e}$).

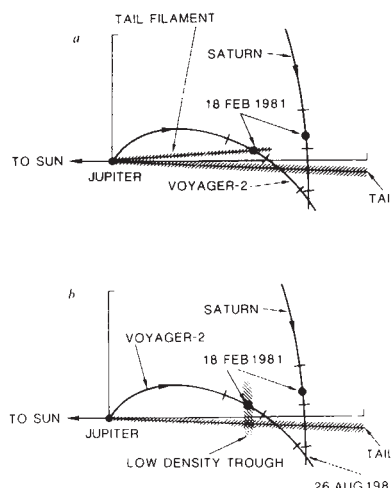


Fig. 2 Paths of Voyager 2 and Saturn with respect to the hypothetical extended tail of Jupiter (a Jupiter-centred Sun-oriented coordinate system is utilized). *a, b*, Different models that could account for the 18 February 1981 detection of continuum radiation from Jupiter's magnetosphere.

As Voyager 2 continued towards Saturn, the ϕ -value reached a maximum in summer 1980, and after that, the spacecraft moved progressively closer to $\phi \approx 175$ – 180° , where the extended tail of Jupiter might again be encountered¹. One of the first very clear events indicative of an actual distant tail traversal occurred in mid-February 1981, and Fig. 1 shows the corresponding wave measurements for the interval 11–22 February 1981; the time profiles of the average electric field strengths detected in 5 of the 16 spectrum analyser channels are displayed. For each channel, the height of the black area is proportional to the logarithm of the electric field strength averaged over a 12.8-min interval, and the distance between the baselines for adjacent channels represents three orders of magnitude in amplitude. The scale on the right-hand side refers to the density that gives an equivalent electron plasma frequency ($f_p = 9,000\sqrt{N_e}$, where N_e is electrons cm^{-3} and f_p is in Hz).

Figure 1 shows that tracking was almost continuous during this 12-day interval and that there were several small but abrupt level changes from one relatively steady amplitude to another. The upper eight channels (1–56 kHz) are mildly affected by a failure in the flight data system^{3,8}, leading to small repetitive or quasi-periodic sequences of changes in background (see, for example, the 1-kHz data for days 43–45). Figure 1 also shows natural (or irregular) changes in levels for the channels from 562 Hz to 5.62 kHz, and the line f_p traces the lower envelope of these variations. We interpret this as detection of continuum radiation from Jupiter's extended tail, and we need to determine whether Voyager 2 was within the tail or connected to it by a solar wind density trough.

These two possibilities are shown in Fig. 2, which uses a Jupiter-centred Sun-oriented coordinate system to show the paths of Voyager 2 and Saturn with respect to the hypothetical

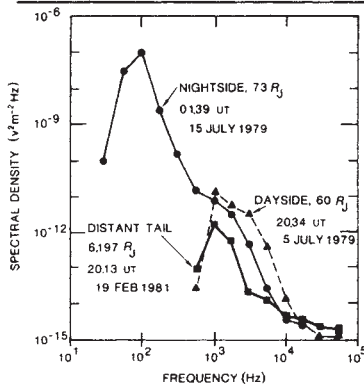


Fig. 3 Spectral densities of continuum radiation detected on Voyager 2 in 1979 (near Jupiter) and 1981 at 6,200 R_J . Low-frequency cutoff varies as the local plasma density changes, but the striking similarities in the spectra at high frequencies suggest Voyager 2 was back in Jupiter's tail early in 1981.

extended tail of Jupiter. Bars on the Voyager and Saturn curves represent the positions on 1 December 1980, 1 April 1981 and 1 August 1981 (more trajectory information is given in ref. 1), and the locations for 18 February 1981 are marked with dots. In mid-February, Voyager 2 was still well above the nominal $\phi \approx 177.5^\circ$ position for an aberrated anti-solar tail configuration, but had the tail fragmented as indicated in Fig. 2a, it is quite likely that Voyager 2 would have been within a tail filament at this time and could have been 'connected' to the tail by a low-density trough as indicated in Fig. 2b.

Voyager 2 plasma probe measurements suggest both phenomena were operative. For the early interval, days 43–48, the spacecraft was apparently immersed in streaming plasma which could have been low-density solar wind, or even a wake region⁹ around a tail filament; the same applies for the end of the interval (days 52, 53). However, for the period centred around 19 February (day 50) when the f_p -profile of Fig. 1 was drawn between $f = 300$ and 500 Hz, the Voyager 2 plasma probe detected no streaming ions, and we propose that at this time the spacecraft was back in Jupiter's tail. Besides the filament hypothesis it is possible that a solar wind disturbance displaced the main tail³.

During the 3-day period which includes this apparent crossing of the distant tail, Fig. 1 indicates that $f_p(\text{min})$ was ~ 400 Hz, leading to $N_e(\text{min}) = 2 \times 10^{-3}$ electrons cm^{-3} . This N_e -value yields a current higher than the plasma probe threshold for ion measurements in the streaming solar wind, but if the flux is lowered because the flow speed is also much reduced, then no plasma will be measured. Thus, the absence of detectable plasma during this period, when N_e was at a minimum is consistent with an actual entrance into the tail cavity.

A final test of the tail-crossing hypothesis involves searching the plasma wave noise spectrum for the characteristics associated with continuum radiation. Figure 3 contrasts characteristic close-in continuum radiation spectra for 5 and 15 July 1979, with a distant tail spectrum from 19 February 1981; for $f \geq 1$ kHz all three spectra have very similar shapes, but at low frequencies there are marked differences. The complete 1979 Jupiter measurements indicate that the continuum radiation has an essentially constant spectral shape throughout the magnetosphere^{6–8}, and that the observed changes are primarily associated with variations in local plasma density, leading to shifts in the low-frequency cutoff. Thus, we associate the 5 July 1979 cutoff at $f \approx 1$ kHz with a local electron density of about 0.01 cm^{-3} , and the $f \approx 50$ Hz cutoff on 15 July 1979 with an extremely low local density value near 3×10^{-5} electrons cm^{-3} . Because for $f \geq 1.0$ kHz, the continuum noise spectrum detected at 6,200 R_J has essentially the same shape as the spectrum measured on 15 July 1979, when Voyager 2 was at 73 R_J on its way to the initial exit from the magnetosphere, this strongly supports the concept that Voyager 2 was again in the magnetosphere. Indeed, Fig. 3 shows that the high-frequency tail spectra from 15 July 1979 and 19 February 1981 differ no more from each other than the two close-in noise spectra. These comparisons suggest that Voyager 2 was within Jupiter's tail on 19 February 1981, and that the tail confines the noise extremely well, or that the continuum radiation is generated within the distant tail.

The demonstration³ that many of the earlier tail-associated events involved leakage of continuum radiation into low-density solar wind troughs argues against an explanation based on noise confinement over a distance of 6,200 $R_J \approx 3$ AU; thus mechanisms that produce continuum radiation far from Jupiter seem more promising. One such process involves production of electromagnetic waves by mode conversion from electrostatic upper hybrid resonance emissions. Observations related to the terrestrial plasmopause, the Io torus boundary, and the perturbations of Saturn's plasma disk at the positions of Tethys, Dione and Rhea¹⁰, suggest that strong mode coupling and intense electromagnetic radiation generally originate in regions with steep plasma density and temperature gradients.

That the magnetopause boundary also has steep gradients in plasma density and temperature suggests that continuum radiation could be generated all along the boundary. The initial Voyager 2 report⁶ clearly showed that intense upper hybrid resonance emissions and weaker continuum radiation were detected immediately after the spacecraft crossed the dayside magnetopause at 71.5 R_J on 5 July 1979. Thus, we speculate that trapped continuum radiation at Jupiter is generated in the region of the magnetopause, so that its appearance at 6,200 R_J does not require strict confinement of the waves. This explanation can also readily account for the similarity in the noise spectra, as Barbosa¹¹ has proposed a wave-scattering mechanism with the spectrum of the continuum radiation controlled by fluctuations of the magnetopause surface.

Discussion

The filament picture in Fig. 2a is consistent with many aspects of the 1981 Voyager 2 measurements but it is useful to consider other sources of information. We can learn much about the possible configurations of extended magnetic tails in the solar wind by studying the structures of comet tails, as far downstream from any interplanetary object the only significant characteristic of the source region involves the close-in size of the obstacle. For visible comets in the inner Solar System, there is no firm knowledge of the true obstacle size, but the theoretical model of Biermann *et al.*¹² gives a contact surface or obstacle radius, R_B , of $\sim 10^5$ km. As the subsolar Jupiter magnetopause is nominally located at $R_B \approx 56 R_J$, this means that the 18 February 1981 measurements were taken at $R \approx 110 R_B$. For the comet case, 110 R_B translates to a downstream distance of about 1.1×10^7 km, and many photographs of the visible ion tails of fresh comets show clear evidence of filaments extending to this distance or beyond (for instance, comet Kohoutek, Fig. 1 in ref. 13). This fact suggests that filaments may develop naturally in extended magnetic tails and that Voyager 2 will continue to encounter them.

If the extended tail of Jupiter has this type of structure, then Saturn itself is likely to have many brief encounters with the jovian tail cavity leading to significant differences between the Voyager 1 and 2 Saturn encounters. It will be of great interest to search Voyager 2 data for such effects, including variations in magnetosphere size, bow shock location, radio emission strength and trapped radiation belt population.

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Stratospheric aerosols properties from Earth limb photography

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Balloon-borne observation at three wavelengths of visible sunlight scattered by the Earth limb allows the determination of aerosols abundances and size distributions at various altitudes in the stratosphere. The stratospheric aerosols are apparently still under the influence of the Mount St Helens volcanic eruption five months after its occurrence on 18 May 1980.

It is only relatively recently that detailed studies of the stratospheric aerosols have been initiated. They are of importance because nucleating and catalytic agents are central in determining the radiation balance of the atmosphere. A survey of light scattering techniques used in the remote monitoring of atmospheric aerosols has been published recently¹. Twilight phenomena have been used both from the ground^{2,3} and from satellites, and Earth-limb observation from space has been based on sunlight scattering observed directly^{4,5} or through its absorption⁶. *In situ* sampling initiated 20 years ago⁷ has also provided much information on the composition of aerosols⁸.

We have previously reported⁹ the photographic observation, from a balloon gondola floating in the upper stratosphere, of the enhancement of stratospheric aerosols over Europe 23 days after the Mount St Helens volcanic eruption. Several other reports have now appeared¹⁰⁻¹³ while details have been provided on the air trajectories which have caused the volcanic plume to move at various altitudes in various directions¹⁴ at the beginning of their many cumbersome revolutions around the Earth. Much information has been collected on the properties of the ejecta in relation to atmospheric effects¹⁵. On 15 October 1980, five months after the eruption, another photographic balloon flight took place. One of the first observations was finding that the enhancement of stratospheric aerosols below 20 km altitude has become very horizontally homogeneous. This means that it is now possible to determine the basic properties of volcanically influenced aerosols and of natural aerosols which seem to differ in several respects in the stratosphere.

Observation method

Photographic cameras onboard a balloon gondola simultaneously record at various wavelengths the light from the Earth limb below the horizontal line-of-sight while the solar elevation is low. This light is scattered direct sunlight with a contribution from the Earth albedo which is minimized when the Sun is low. The observation geometry is shown in Fig. 1. The limb radiance, R , can be expressed directly in solar radiance units because solar images are recorded simultaneously with a known attenuation factor. The solar elevation is calculated from the time that the picture was taken and from the geographical position. An angular scale is constructed from several consecutive shots. The depression angles at which radiances are measured are related to the line-of-sight altitude of closest approach to the Earth surface taking into account refraction effects¹⁶. The measured integrated radiances can be inverted taking into account absorption by ozone and air along the line of sight by the 'onion peeling' method to yield the *in situ* radiance, R^* , per unit length versus altitude.

The Sun-oriented gondola can be rotated about its vertical axis so that pictures can be taken at various azimuth angles, A , relative to the Sun's position. The scattering angle θ of direct

solar radiation can be computed from the relationship

$$\cos \theta = -\sin D \sin h_{\odot} + \cos D \cos h_{\odot} \cos A \quad (1)$$

where D is the depression angle at which the atmospheric radiance is measured and $h_{\odot}$ is the solar elevation angle at the time of measurement. As expected from their strong forward scattering properties, aerosols re-emit little light at an azimuth angle 180° away from the direction of the Sun. The limb radiance observed in this case is used to subtract Rayleigh scattering and to isolate aerosols scattering at all azimuths taking into account the Rayleigh phase function¹⁷.

If direct solar radiation only is considered, an *in situ* monodisperse aerosol radiance R_a^* per cm of pathlength is related to the average solar radiance $R_{\odot}$ by

$$R_a^* = R_{\odot} \pi \alpha^2 Q_s \sigma \varphi n / 4\pi \quad (2)$$

where α is the angular radius of the Sun's disk, 2.16×10^{-5} rad, φ the properly normalized particulate phase function, Q_s the scattering efficiency factor, σ the particulate scattering geometrical cross-section in cm^2 if the number density, n , of the particles is expressed in cm^{-3} .

In practice, our data confirm the previous observation¹⁸ according to which the phase function can, within experimental uncertainties, be represented by the Henyey-Greenstein function. The asymmetry factor g of the phase function and the total scattering efficiency can then be determined. The variation

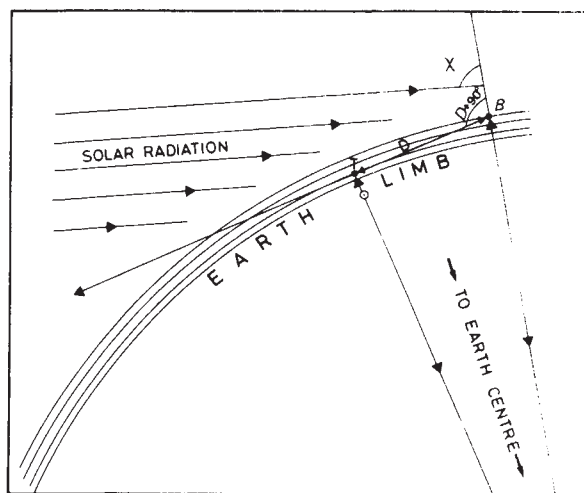


Fig. 1 Observational geometry: quasi parallel light falls on the atmosphere at a solar zenith angle χ , the atmosphere is seen at various depression angles D ; through the rotation of the gondola, photographs are taken at various azimuth angles A from the Sun direction.

of these parameters with wavelength can be fitted to their theoretical variations using the effective size parameter of Hansen and Travis¹⁹. This procedure, which uses the Mie scattering computation programme of Wiscombe²⁰, leads to an evaluation of the effective particle size and provides information on other properties. From these two quantities and from the product $n\sigma$ obtained directly from equation (2) the value of n can be deduced.

The aerosol particle size is usually treated in terms of the wavelength, λ , of the interacting light through the Mie size parameter

$$x = 2\pi a/\lambda \quad (3)$$

where a is the radius of the spherical particle in a monodisperse aerosol. In a polydisperse aerosol the number of particles of radius r is expressed¹⁹ by

$$n_r = Ar^{(1-3b)/b} e^{-r/ab} \quad (4)$$

where a becomes the effective radius while b is a measure of the width of the size distribution; A is a constant related to the total number of particles.

Comparing R_a^* with θ , the scattering angle, at various wavelengths provides information about the asymmetry factor, g . A knowledge of g and its variation with the wavelength of the interacting light leads to the unique determination of a and b . How the scattering efficiency Q , varies with size parameter can then be determined. The behaviour of these quantities is known from the Mie scattering theory.

Experimental details

The balloon flight took place on 15 October 1980, the tropopause height being 11.4 km. The gondola floated at 37.6 km altitude and was equipped with seven Hasselblad EL 500 cameras with 80-mm focal length lenses and 70-mm films. Four of them were pointing forwards, one being loaded with colour EPR 475 Kodak film, and three in the opposite direction. The

six black and white cameras contained (each camera was paired with the one opposite): Wratten filters 47 (440 nm) and 25 (650 nm) with Plus X Kodak film and filters 87 with Kodak aerographic 2424 film (860 nm). To ensure that the Sun images were in the dynamic range of the film, neutral density screens made of exposed and processed sheet films were placed 60 cm from the lenses with their lower edge placed a few centimetres above the optical axis. Before flight, step wedges, inconel filters and samples of the neutral density screens illuminated in parallel light were recorded by each camera in the laboratory. After the flight, the Plus X films were processed using the Kodak D76 developer ($\gamma = 1$) and the IR-sensitive film was processed in the Kodak D19 developer ($\gamma = 2.3$). The dynamic range of this last film was too small to record usefully simultaneous Sun and limb images so that its absolute calibration was derived from the comparison of optical densities due to quasi-pure Rayleigh scattering observed 180° from the Sun in the three colours above 30 km altitude. The absolute calibration in blue and red light was based directly on solar images. The colour, blue, red and IR camera settings (aperture, speed) were respectively: $f/11$, $1/125$ s; $f/8$, $1/250$ s; $f/8$, $1/250$ s; $f/8$, $1/125$ s. As soon as the gondola could be Sun oriented, pictures were taken during ascent and later during the float period. With its Sun sensor remaining locked on the Sun, the gondola was then rotated about its vertical axis so that the cameras could record Earth limb images 65°, 115°, 180°, 245° and 295° from the solar azimuth. The whole horizon scan took place between 16.08 and 16.18 h GMT, during which atmospheric illumination conditions changed very little. The latitude and longitude were respectively 1° E and 44° N. The average solar azimuth and elevation angles were 248° and 9.3° respectively. The optical densities were measured on three tracks per frame normally to the horizon by means of a Jarrell-Ash micro-densitometer and subsequently converted into radiance, in units of solar radiance, versus altitude of the grazing line of sight every 200 m. Each photograph allows an horizontal angular coverage of about 34°.

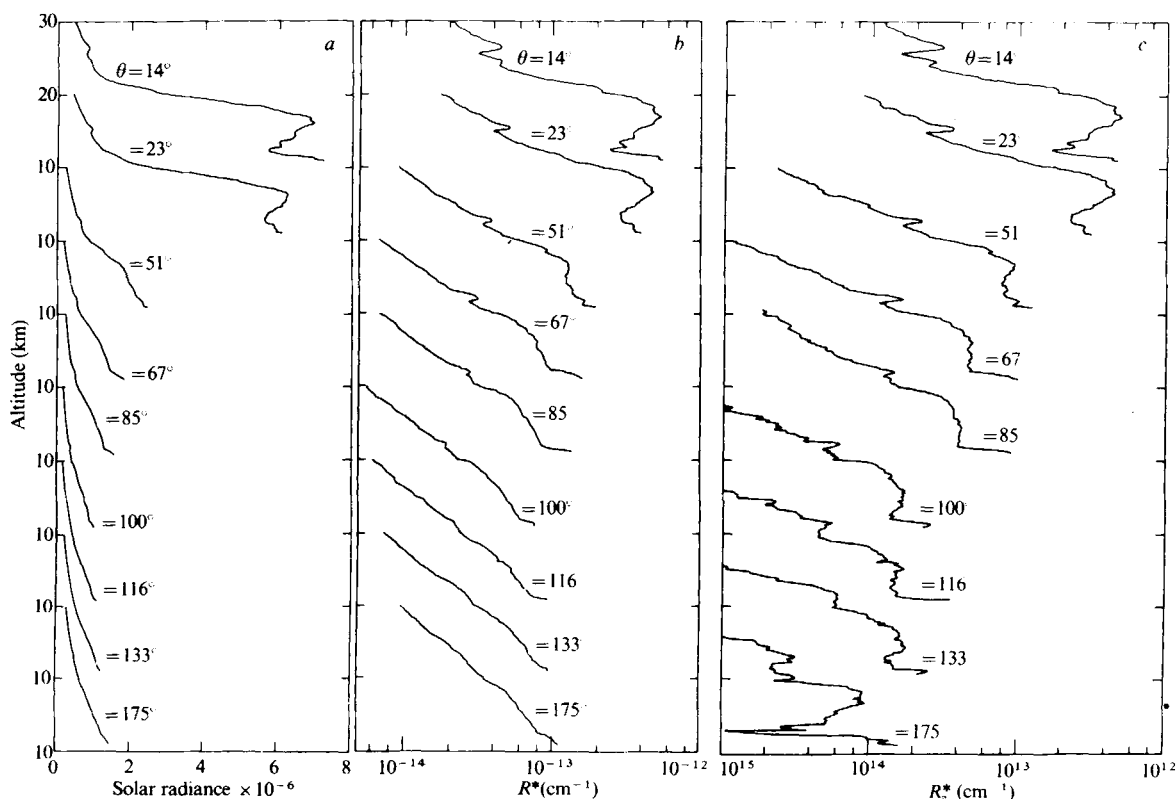


Fig. 2 Data at 650 nm versus altitude for various scattering angles, θ : *a*, integrated radiances, R , along the lines of sight; *b*, inverted radiances, R^* ; *c*, inverted radiances due to aerosols, R_a^* . Each successive lower curve is displaced by 10 km altitude from the one above. R^* and R_a^* are expressed in units of solar radiance.

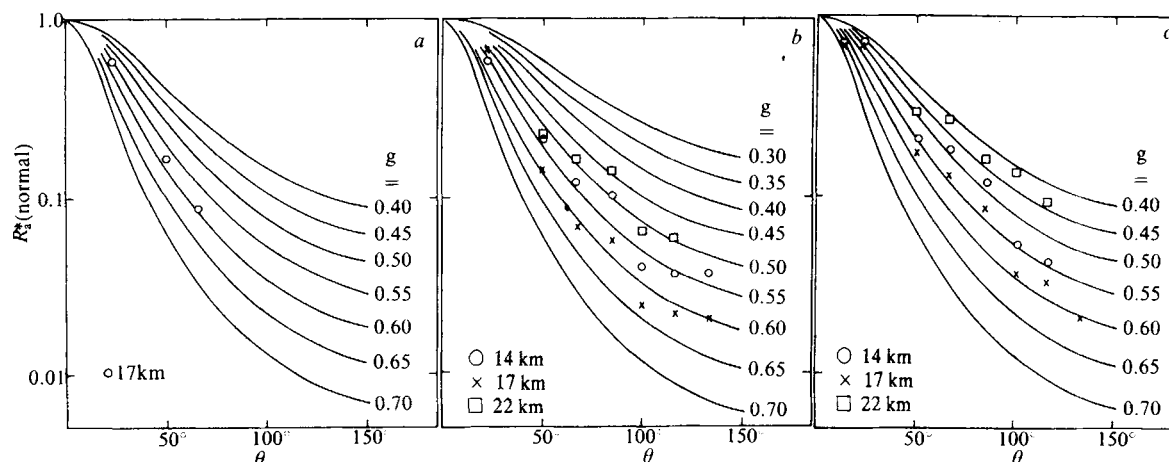


Fig. 3 Henyey-Greenstein phase functions of the radiance versus scattering angles, normalized at $\theta = 0^\circ$ for various values of the asymmetry factor, g . The experimental data points are shown at the three altitudes studied and for the three wavelengths: a, 440 nm; b, 650 nm; c, 860 nm.

Results

As an example, the observed limb radiance values are presented versus the altitude of closest approach of the line of sight to the Earth surface (sea level) for 650 nm and for various scattering angles in Fig. 2a.

The comparison of the vertical radiance profiles observed at small scattering angles in red light 11 days before, less than 1 month⁹ and five months after the Mount St Helen's eruption shows several characteristics. A radiance enhancement peaking at 17 km altitude still exists on 15 October 1980, by a factor of almost three relative to the pre-eruption value. Above 22 km altitude the radiance has returned to values close to what it was on 7 May 1980. This suggests that the heavy layer observed over England on 6 and 7 June¹⁰ and on 5 June⁹ over France constituted the main body of the material injected in the stratosphere by the volcano. Over 5 months, that layer would then have spread in altitude and its centre of gravity would have moved upwards by ~ 2 km, this being perhaps related to air heating through sunlight absorption by the aerosols.

The various photographs taken around the vertical axis of the gondola reveal the 17 km layer at all azimuth angles; above 20 km altitude a well defined 1-km thick layer is readily seen. Such thin, well separated features have been observed on each of our previous flights and most probably have no relationship with volcanic activity. They belong to the 'natural' stratospheric aerosol. In this particular case the layer is present towards the south from the gondola and absent towards the north. Its altitude is at 22 km from 125° azimuth counted clockwise from the geographical north to $\sim 220^\circ$ where it begins to rise up to ~ 25 km at 250° where it splits and becomes invisible. Particulate matter is also present above, what we call here, the 22 km layer. With respect to the horizontal homogeneity, the comparison of the aerosol direct sunlight scattering with the scattering angles is safe for the 17-km layer; for higher altitudes care must be taken.

For the angular study, along the lines-of-sight integrated radiances are inverted using standard ozone and air distributions²¹ and their respective absorption cross-sections^{17,22}. The *in-situ* radiance values, R^* , in units of solar radiance and per cm along the line-of-sight at the tangent altitude, are presented in Fig. 2b. The R^* values at 650 nm (Fig. 2b) show at $\theta = 175^\circ$ an exponential dependence versus altitude, as expected, on which some structure and noise is superimposed. At 860 nm in the backward hemisphere where aerosols are expected to bring a small contribution and particularly at 30 km altitude, the variation of R^* with θ fits well the Rayleigh phase function, giving confidence to the very low contribution from the Earth albedo to the observed signal. This is not surprising as the average cloud deck radiance measured at depression angles from 5 to 15° ranges from 7×10^{-7} to 6×10^{-7} of the solar

radiance at respective azimuths from 0 to 180° from the Sun. At $\theta = 14^\circ$ and 17 km altitude the aerosol contribution is about 14 times the Rayleigh contribution.

At 650 nm (Fig. 2b) the Rayleigh phase function can be fitted to the radiance variation observed in the backward hemisphere at 30 km altitude. In this case, however, the average cloud radiance varies from 6×10^{-6} near the Sun's azimuth to 6×10^{-7} elsewhere. In the forward direction the aerosol contribution to the radiance is 10.8 times the Rayleigh contribution at 17 km. At 440 nm, Rayleigh scattering dominates even if at $\theta = 14^\circ$ it still seems to be five times smaller than aerosol scattering at 17 km. At $\theta = 175^\circ$, R^* grows faster than exponentially at altitudes below 20 km. Multiple scattering here has an important role because the Rayleigh optical thickness on the tangent line of sight reaches unity at 20 km. This occurs at 9 km and at ground level for 650 and 860 nm respectively. In addition the average cloud deck radiance is very high in blue light varying from 1.6×10^{-5} near the Sun to 10^{-6} of the solar radiance elsewhere. The data obtained in blue light can then only be used

Table 1 Summary of balloon-borne results for three characteristic altitudes

Altitude	14 km	17 km	22 km
g_{440}	—	0.6	—
g_{650}	0.55	0.60	0.50
g_{860}	0.52	0.56	0.45
$\sum Q_s n \sigma_{440} (\text{cm}^{-1})$	—	3.2×10^{-8}	—
$(\sum Q_s n \sigma_{440})_{AM} (\text{cm}^{-1})$	—	1.5×10^{-8}	—
$\sum Q_s n \sigma_{650} (\text{cm}^{-1})$	9.4×10^{-9}	1.0×10^{-8}	2.9×10^{-9}
$(\sum Q_s n \sigma_{650})_{AM} (\text{cm}^{-1})$	6.0×10^{-9}	5.8×10^{-9}	1.5×10^{-9}
$\sum Q_s n \sigma_{860} (\text{cm}^{-1})$	1.9×10^{-9}	2.7×10^{-9}	9.3×10^{-10}
$(\sum Q_s n \sigma_{860})_{AM} (\text{cm}^{-1})$	2.4×10^{-9}	3.1×10^{-9}	9.2×10^{-10}
Q_{s440}	—	0.8	—
Q_{s650}	0.14	0.52	0.1
Q_{s860}	0.10	0.35	0.05
$\sum n \sigma_{440} (\text{cm}^{-1})$	—	4×10^{-8}	—
$(\sum n \sigma_{440})_{AM} (\text{cm}^{-1})$	—	1.9×10^{-8}	—
$\sum n \sigma_{650} (\text{cm}^{-1})$	6.7×10^{-8}	1.9×10^{-8}	2.9×10^{-8}
$(\sum n \sigma_{650})_{AM} (\text{cm}^{-1})$	4.3×10^{-8}	1.1×10^{-8}	1.5×10^{-8}
$\sum n \sigma_{860} (\text{cm}^{-1})$	1.9×10^{-8}	7.7×10^{-9}	8.9×10^{-9}
$(\sum n \sigma_{860})_{AM} (\text{cm}^{-1})$	2.4×10^{-8}	8.9×10^{-9}	8.8×10^{-9}
$a (\mu\text{m})$	0.042	0.15	0.045
b	3.6	0.6	1.8
$V (\text{cm}^3)$	1.8×10^{-13}	1.5×10^{-13}	7.0×10^{-14}

g , The asymmetry factor of the phase function; $\sum Q_s n \sigma$, the optical aerosol scattering extinction deduced from observed radiances; $(\sum Q_s n \sigma)_{AM}$, the optical aerosol scattering extinction deduced from comparison with the molecular scattering extinction; Q_s , the scattering efficiency factor; $\sum n \sigma$ and $(\sum n \sigma)_{AM}$, the geometric scattering extinctions; a , b , the size distributions parameters; V , the total volume of particles per cm^3 of air at the respective altitudes.

with caution and at small scattering angle. The situation here is different from that in the balloon-borne aureole observation²³ due to the low solar elevation used.

Figure 2c presents the *in situ* radiance due to aerosols R_a^* obtained by subtracting the Rayleigh scattering R_M^* , due to air. R_M^* is evaluated from R^* at $\theta = 175^\circ$ following the 'clean air' procedure currently used in lidar work²⁴. R_M^* is then adapted for the various scattering angles considered according to the molecular phase function¹⁷.

Interpretation

The angular dependance of R_a^* has been fitted to the variation of the scattered light intensity with θ computed for various values of the asymmetry factor g of the Henyey-Greenstein function as shown for three wavelengths in Fig. 3. In blue light a few values of θ give useful results. For the reasons discussed above values for the 22-km layer have only been considered for θ between 51° and 116° .

The asymmetry factors so determined are listed in Table 1 with the optical scattering extinction coefficients $Q_s n \sigma$. The values at 440 nm have not been used because a model taking into account the measured cloud radiances indicates a non-negligible contribution of the albedo to the limb radiance. But this contribution is negligible at the larger wavelengths. The observed g values, taking into account that it is probably slightly higher than 0.6 and 440 nm, tend to indicate that a value of the real index of refraction equal to 1.55 (see ref. 25) is observed here. This value will then be used to deduce the size parameter x , and its variation with λ from 650 to 860 nm leads to the values of a and b characterizing the size distribution listed in Table 1. Eventually, the scattering efficiency Q_s can be deduced from a and b following the Mie scattering theory¹⁹.

With the data available, there is, of course, another method of determining $\sum Q_s n \sigma$. From the air σ values¹⁷ and from the air number density n_{air} taken from a model²¹ (mid-latitude, spring-autumn) ($n \sigma$)_{air} can be evaluated. The R_M^* values determined at $\theta = 175^\circ$ by the 'clean air' method²⁴ are compared with the R_a^* measured and by taking into account the air and aerosols phase

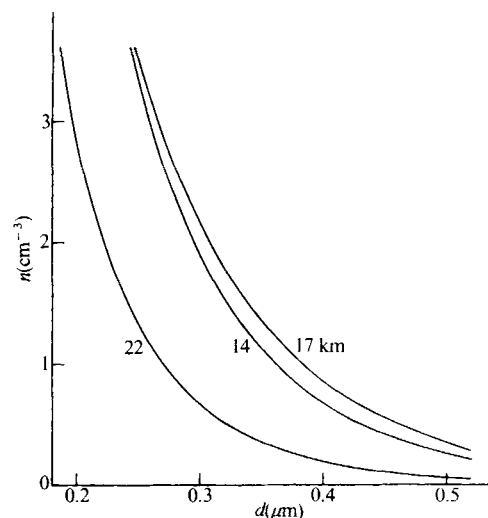


Fig. 5 Total numbers of particles with diameters larger than given values. The ratio of the number of particles with diameters (d) $> 0.3 \mu\text{m}$ to the number of particles with diameters $> 0.5 \mu\text{m}$ is larger for the layer at 22 km altitude than for 17 and 14 km altitudes indicating the volcanic influence in these two latter cases²⁹.

functions the values of $(\sum Q_s n \sigma)_{\text{AM}}$ for the aerosols are deduced. They are also listed in Table 1.

The absolute particle distribution shown in Fig. 4 for the three altitudes considered is based only on the geometrical extinction coefficient $\sum n \sigma$ at 860 nm for two reasons. In IR light, the albedo contribution to the measurement is negligible while it is a maximum in blue light and in the inversion of R leading to R^* the correction for the absorption by O_3 , of which a standard vertical distribution had to be used, is the largest in red light. This is supported by the discrepancy observed at shorter wavelength between the extinctions determined from the observed radiances and those determined by comparison with the molecular scattering. The agreement is very good at 860 nm. The Mie scattering theory also had the best chance to be valid, even if the particles do not exhibit a perfectly spherical and smooth shape, at small values of the size parameter.

Discussion

Our particle distributions are compared in Fig. 4 with the upper and lower limits deduced from *in situ* sampling⁷ and with two extreme values resulting from solar aureole measurements²³. The present results fall within the range of those data except for particles $< 0.2 \mu\text{m}$ radius where the upper limit given by Junge *et al.*⁷ is exceeded. As mentioned above the aerosols below 20 km are still in volcanically perturbed conditions. On the other hand, the collection efficiency of an *in situ* sampler might be reduced for small particles. Note also that our distributions are steeper especially at large radii. This effect is less pronounced in the comparison with the aureole data. This effect seems to be due to the small values of effective radii obtained here.

Another comparison of our data, shown in Fig. 5, can be made for the total number of particles with a radius $> 0.15 \mu\text{m}$ obtained by balloon-borne optical particle counters²⁶. The number densities of these particles are $0.4\text{--}1.3 \text{ particle cm}^{-3}$ in the 14–17 km altitude region and $0.1\text{--}0.9 \text{ particle cm}^{-3}$ at 22 km. The data presented here for 22 km correspond to a low aerosol content above 20 km and lead to $0.7 \text{ particle cm}^{-3}$ above $0.15 \mu\text{m}$. At 14 and 17 km altitude the values presented in Fig. 5 respectively imply 1.9 and $2.1 \text{ particle cm}^{-3}$ of particles with radii $> 0.15 \mu\text{m}$. A comparison between the two methods would be required to define a possible discrepancy as the aerosols are volcanically enhanced in the present case.

The optical extinction coefficient $Q_s n \sigma$ measured at $1 \mu\text{m}$ wavelength by the SAM II satellite-borne instrument at low

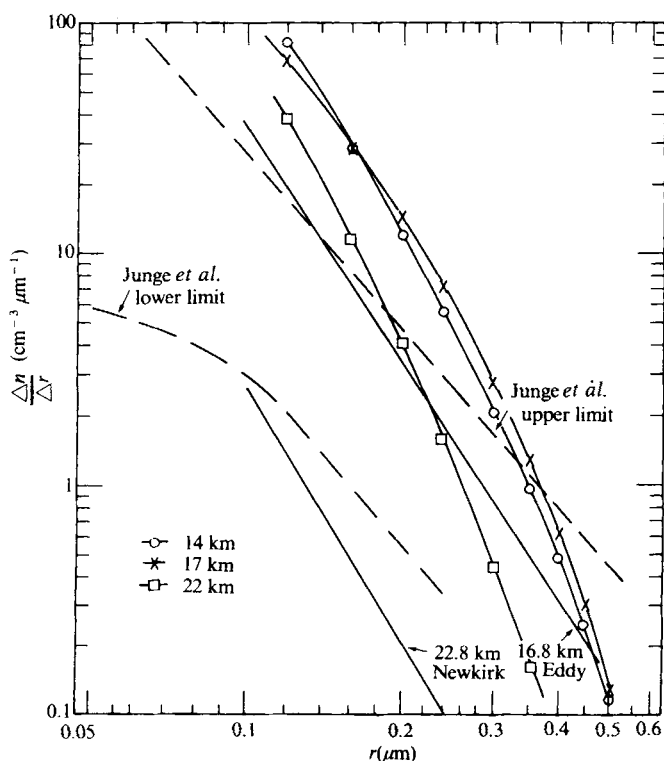


Fig. 4 Number of particles per cm^{-3} versus their radii and per micrometre size interval for three characteristic altitudes: 14, 17 and 22 km. Two previous data sets^{7,23} are shown for comparison.

aerosol load in July 1979²⁷ exhibits an almost constant value at altitudes from 14 to 20 km of 10^{-9} cm^{-1} . This compares very favourably with our 860 nm value at 22 km. At 17 and 14 km altitude, the extinction in the present case is 3 and 2 times larger. This can be expected from the volcanic influence which may also explain the larger effective particle radius a at 17 km altitude.

A value of the asymmetry factor g of 0.49 ± 0.7 has been measured recently at 633 nm, a wavelength close 650 nm used here, from 10.7 to 12.8 km altitude in the stratosphere¹⁸. This value corresponds to our measurement at 22 km where the aerosol is considered to be purely 'natural'. Because g is larger in the present case at 14 and particularly at 17 km, one of the volcanic influences must then be to increase the g value and if the Mie theory is applicable to increase the particle size as we observe it at 17 km altitude.

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From an optical point of view the particle size distribution, of which the shape is of greater interest than the particle concentration for radiative transfer calculations²⁵, is variable in the stratosphere. The asymmetry factor, g , is smaller than the constant value (0.7) currently used in models.

For photochemical models of stratospheric aerosols²⁸ the variable size distribution will have a part to play. The molecular content of the condensed phase thought to be oxidized sulphur, water vapour and perhaps other species is not negligible for the gas phase either as conversion into vapour would lead to volume concentrations in the 10^8 – 10^9 molecules cm^{-3} at altitudes from 15 to 20 km.

Our measurements therefore confirm recent data and provide new information on critical points which are of fundamental importance in the evaluation of the role that stratospheric aerosols in the size range from 0.04 to 0.4 μm radius can play.

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Extrachromosomal circular copies of the eukaryotic transposable element *copia* in cultured *Drosophila* cells

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Circular copies of the transposable element copia have been isolated from cultured Drosophila cells. These molecules, which are present at approximately 1 copy per 10–50 cells, comprise a heterogeneous family of related species. Most are composed of a complete copy of the internal section of copia combined with either one or two copies of the flanking direct repeat sequences. Such structures are strikingly analogous to the circular proviral forms of retroviruses.

TRANSPOSABLE genetic elements were first defined genetically in maize¹ and have since been found in a wide variety of organisms including bacteria^{2,3}, yeast³ and *Drosophila*^{3–5}. They are capable of causing a wide range of genetic effects such as abnormally high mutation and reversion rates and chromosomal deletions or rearrangements. Recent experiments indicate that most, if not all, of these effects are connected with the ability of these elements to transpose to different sites in their host cell genomes. Although there is no direct evidence for analogous species in vertebrates, several observations strongly suggest that eukaryotic transposable elements and the integrated proviral forms of some retroviruses are related.

The following properties are shared by all eukaryotic transposable elements and integrated provirus retroviruses^{6–14}:

- Their sequences are each composed of an internal DNA segment several thousand nucleotide pairs long flanked by a pair of identical DNA segments of several hundred base pairs (bp) which are both arranged in the same direct orientation on the element^{6–14} (Fig. 1). These repeat sequences are each bounded by small inverted repeats several nucleotide pairs long^{7–13}.

- The complete element is flanked by an identical pair of host DNA sequences, usually 4–6 bp long, that are present only once in the target site for integration^{7,10–12,14}.

- There is no detectable homology between the DNA sequences at the insertion site and the ends of the element.

- The terminal dinucleotides of a transposable element or integrated retrovirus provirus are 5'TG...CA3' and in some cases this sequence homology extends further into the elements,



Fig. 1 Generalized structure of a eukaryotic transposable element or integrated retrovirus provirus. An internal segment of several kilobases is bounded by a pair of identical directly repeated sequences. These repeats carry small terminal inverted repeats several base pairs long which are normally imperfect ($\leftrightarrow$ and $\leftarrow$). The complete element is flanked by an identical pair of host DNA sequences ($\blacktriangleright$). The flanking genomic DNA is indicated ($\sim$).

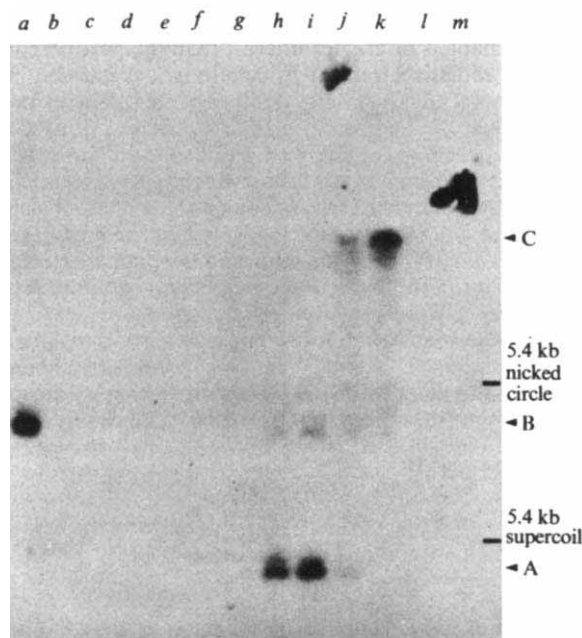


Fig. 2 Isolation of extrachromosomal *copia*-specific DNA. Circular DNA was extracted from *Drosophila* cultured cells (Eschaler's Kc line²⁸; 1.5×10^9 cells) by a modification of the method of Hirt^{17,29}. The enriched sample was then subjected to CsCl-ethidium bromide ultracentrifugation. Nucleic acids were recovered from the fractionated gradient, dissolved in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and treated with boiled RNase A ($10 \mu\text{g ml}^{-1}$ at 37°C for 15 min). 30% of each fraction was assayed for *copia*-specific sequences by agarose gel electrophoresis in 90 mM Tris-OH, 90 mM boric acid and 9 mM EDTA at 1.5 V cm^{-1} 16 h, transfer to a nitrocellulose filter²⁰ and probing with ^{32}P -labelled nick-translated *copia* DNA³⁰ (a 5.1-kb *MspI* fragment of the genomic clone cDm2056¹⁴ containing the entire *copia* element plus approximately 150 bp of flanking material). Dextran sulphate (9%) was used during hybridization³¹. The filter was exposed to Fuji Rx film with an intensifying screen at -70°C for 16 h. Lane a, 50 pg of the linear 5.1-kb *copia MspI* fragment run in parallel, lanes b-m, gradient fractions 1-12 (fraction 1 is the bottom). The mobilities of supercoiled and nicked circular forms of the marker 5.4-kb plasmid pJB8 (ref. 29) are shown. Class A-C molecules indicate *copia* species which are described in the text.

for instance the *Drosophila* element *copia* shares with an avian retrovirus the sequence 5'TGT...TACAACA3' (refs 7, 13).

Although such shared properties are very unlikely to be coincidental, the exact relationship between transposable elements and integrated retrovirus proviruses is unclear. To probe further the similarities between eukaryotic transposable elements and retrovirus proviruses, we have searched for transposable element-encoded structures which are analogous to intermediates in the retroviral life cycle. One such intermediate is the circular double-stranded DNA copy of the viral RNA that is synthesized by the viral reverse transcriptase and is presumed to integrate into the host cell genome. The present report describes the isolation and characterization of closed circular DNA copies of the *Drosophila* transposable element *copia* whose structures closely resemble the circular forms of retrovirus proviruses. This observation further demonstrates the remarkable similarity between these classes of element.

Isolation of circular *copia* DNA molecules

Extrachromosomal circular DNA was first described in *Drosophila* by Stanfield and Helinski¹⁵. *Drosophila* eggs and cultured cells contain a heterogeneous population of circular species with sizes varying from a few hundred nucleotides to 20 kilobases (kb). More than 80% of this DNA is homologous to middle repetitive DNA¹⁶ (sequences repeated ~ 100 times in the *Drosophila* haploid genome) of which the *copia* element is an example. It therefore seemed plausible that *copia* might be represented in *Drosophila* circular DNA.

Circular DNA was isolated from *Drosophila* culture cells by a modification of the method of Hirt¹⁷. The partially purified circles were separated from contaminating genomic DNA by CsCl-ethidium bromide isopycnic ultracentrifugation and the fractions from the gradient were assayed for *copia* sequences by agarose gel electrophoresis and Southern filter hybridization¹⁸. Three major size classes of *copia*-specific sequence were observed (Fig. 2): a fast migrating class (class A) which is shown below to comprise circular supercoiled molecules, minor amounts of nicked circular molecules with intermediate mobility (class B) and a slowly migrating class with a lower buoyant density containing high molecular weight genomic linear DNA (class C). The following experiments proved the assignments for class A and class B molecules; class C molecules were not studied further.

First, class A co-purifies with supercoiled mitochondrial DNA on the CsCl-ethidium bromide gradient (data not shown), strongly suggesting that class A is also supercoiled. Further evidence was provided by digestion of class A and class B molecules with restriction endonucleases. Gradient fractions containing the fast migrating class A *copia* species were pooled and digested with restriction enzymes which cut genomic *copia* elements once (*HindIII*) or not at all (*MspI*; Fig. 3). Mock digestion with no added enzyme led to partial conversion of class A (which in Fig. 2 is resolved into a doublet) into the more slowly migrating class B. DNA preparations of this purity usually contain low levels of contaminating nuclease which nick supercoiled circles to yield open circles. Thus, in the conditions of mock digestion, supercoiled class A circles were converted

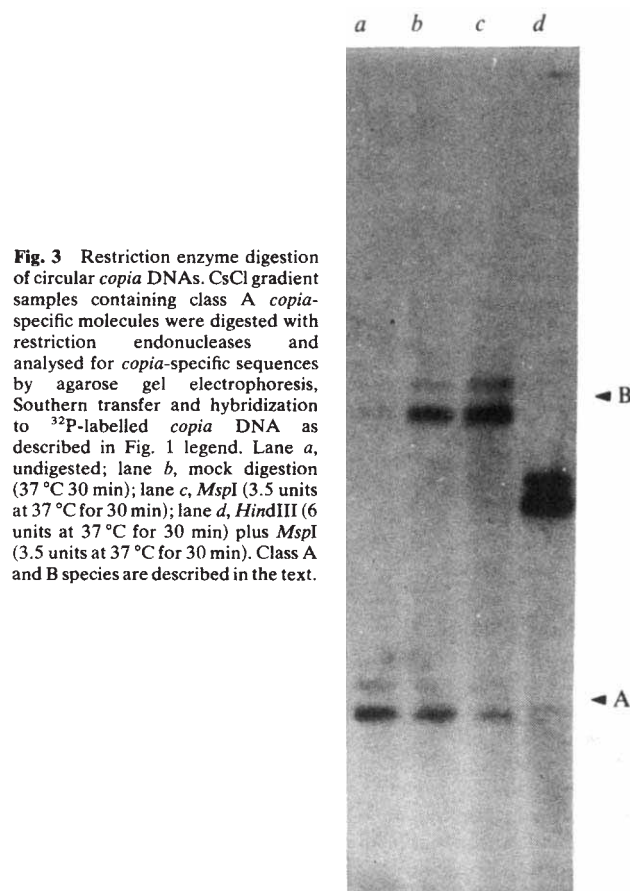


Fig. 3 Restriction enzyme digestion of circular *copia* DNAs. CsCl gradient samples containing class A *copia*-specific molecules were digested with restriction endonucleases and analysed for *copia*-specific sequences by agarose gel electrophoresis, Southern transfer and hybridization to ^{32}P -labelled *copia* DNA as described in Fig. 1 legend. Lane a, undigested; lane b, mock digestion (37°C 30 min); lane c, *MspI* (3.5 units at 37°C for 30 min); lane d, *HindIII* (6 units at 37°C for 30 min) plus *MspI* (3.5 units at 37°C for 30 min). Class A and B species are described in the text.

partially into nicked class B circles. Digestion with *MspI* gave a similar result but addition of *HindIII* to this enzyme converted both classes of species into a doublet of slightly greater mobility than class B, presumably corresponding to linear molecules. We conclude that classes A and B in Fig. 2 are composed of supercoiled and nicked *copia* circles, respectively. Furthermore,

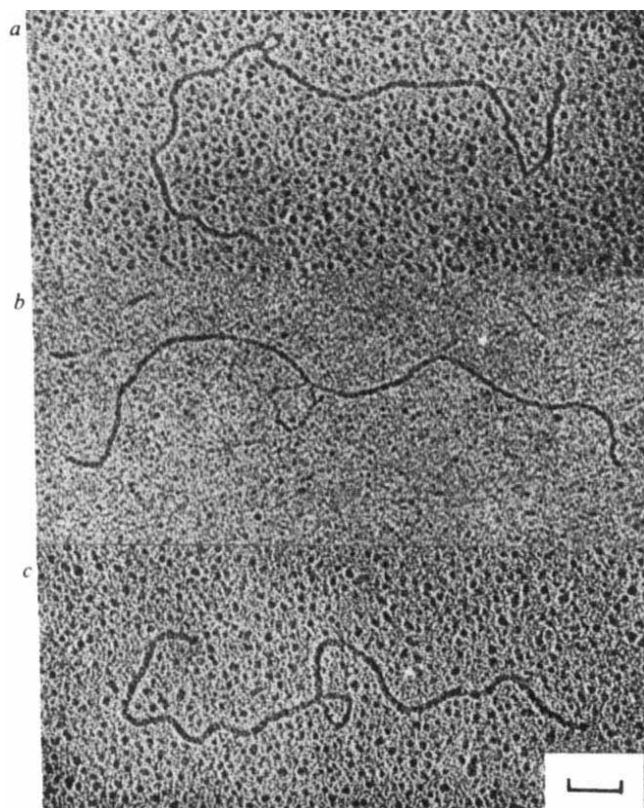


Fig. 4 Plasmids containing 5.0-kb (pBB1 and pBB5), 4.7-kb (pBB3) and 4.2-kb (pBB6) inserts were digested with *Hind*III and *Msp*I restriction endonucleases to yield the linear insert DNAs (*Msp*I cuts the plasmid pAT153 to yield fragments smaller than 700 bp). Heteroduplexes between pair-combinations of these inserts were prepared and mounted³². *a*, 5.0-kb and 4.7-kb inserts; *b*, 5.0-kb and 4.2-kb inserts; *c*, 4.7-kb and 4.2-kb inserts. The bar indicates 0.1 μ m.

visualized by electron microscopy (Fig. 4). Deletion loops were only observed between plasmids of different sizes, suggesting that the two members of each size class possess substantially similar sequences. Only single deletion loops were observed between plasmids containing inserts of different sizes, and the sizes of the loops correspond in all cases to the size difference between each pair of inserts. It therefore seemed likely that the 4.7-kb insert was a simple deletion variant of the 5-kb species and that the 4.2-kb species had a larger deletion in the same region.

Restriction mapping of the five plasmids (Fig. 5) confirmed this conclusion. All three classes of insert have similar restriction maps, the only difference between them being in the region between the unique *Pst*I and *Pvu*II sites. The 5.0-kb inserts have the restriction map predicted for an excised genomic *copia* element which has been circularized and cloned. In particular, these inserts contain the 280-bp *Bal*II fragment predicted from the head-to-tail fusion of two direct repeats. The 4.7-kb inserts lack this *Bal*II fragment and presumably contain only one direct repeat. The 4.2-kb insert has lost both the *Bal*II fragment and a further 500 bp to the left of it.

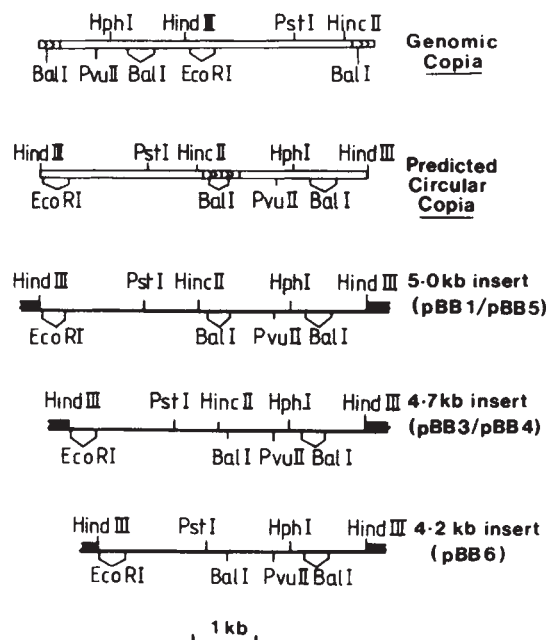


Fig. 5 Restriction maps of cloned *copia* circular DNA molecules. DNA of the vector phage λ Charon 21A (a gift of R. A. Flavell) was treated with restriction endonuclease *Hind*III (Boehringer; fourfold overdigestion) and calf intestinal phosphatase (Boehringer, 0.02 U per μ g DNA for 30 min at 37 °C). The enzymes were inactivated at 70 °C for 60 min. Enriched circular DNA from *Drosophila* cultured cells (see Fig. 1 legend) was digested with *Hind*III and *Msp*I restriction endonucleases, extracted with phenol and precipitated with ethanol. The cleaved vector DNA (2 μ g) and insert DNA (~0.01 μ g) were ligated in 0.01 ml 50 mM Tris-HCl pH 7.5, 10 mM dithiothreitol, 5 mM $MgCl_2$ and 1 mM ATP with T_4 DNA ligase (a gift from A. Udvardy) at 4 °C for 16 h. The mix was packaged into phage coats³³ and phage were plated on *Escherichia coli* K802 cells. Duplicate nitrocellulose filters were blotted from the plates³⁴ and hybridized to ³²P-labelled *copia* DNA as for Southern filters. Thirteen plaques showing hybridization were picked and the phage plaque-purified three times. Quick DNA preparations were made from suspension stocks of the phage³⁵. Insert DNAs from these phages were sized by agarose gel electrophoresis of *Hind*III digests. Eight phage contained 4.7-kb inserts, three contained 5.0-kb inserts, one contained a mixture of these two and one contained a 4.2-kb insert. Two examples of both the 4.7-kb and 5.0-kb inserts, together with the 4.2-kb insert, were subcloned into *Hind*III-digested pAT153²⁰ and the resultant recombinants were used to transform HB101 cells to ampicillin resistance. Quick plasmid DNA preparations and large-scale preparations were by the method of Ish-Horowitz and Burke²⁹. A restriction map of a genomic *copia* element (G. Rubin, unpublished) is shown and the location of the direct repeats (■) is indicated. The predicted restriction map of a *copia* element, which has been perfectly excised from the recombinant plasmid cDm 2056¹⁴, cyclized and then cleaved at the unique *Hind*III site, is shown. Double cutting sites for *Bal*II and *Eco*RI enzymes are indicated (∩).

there are at least two different sizes of circle, the smaller predominating.

The approximate yield of circular *copia* DNA recovered on the CsCl gradient was determined by comparison with known quantities of cloned *copia* DNA (Fig. 2). The hybridization signal from the circular *copia* DNA corresponds to about 50 pg of DNA from 4×10^8 cells, which is equivalent to 1 complete *copia* molecule per 50 *Drosophila* cells, assuming complete recovery. The actual recovery of circular DNA is difficult to establish but is likely to be ~25% (the value obtained from similar experiments by Smith and Vinograd)¹⁹, suggesting that the cultured cells studied contain 1 circular *copia* molecule per 10–50 cells.

Structure of cloned *copia* molecules

To analyse circular *copia* molecules more conveniently, these species were cloned into a bacteriophage λ vector. The circles were linearized by digestion with *Hind*III and then ligated into *Hind*III-cleaved λ Charon 21A. Approximately 1,000 *copia*-specific recombinant phage were detected; 13 of these were plaque-purified and phage DNA preparations were made. The size of the *copia*-specific DNA insert was determined in each of these purified phage by *Hind*III digestion followed by agarose gel electrophoresis. Two major size classes of insert were observed, 4.7 and 5.0 kb long. These two species are presumably derived from the two major size classes of *copia* circle visible in Fig. 2. Two examples of each of the size classes of recombinant, together with one phage containing a smaller 4.2-kb insert, were subcloned into plasmid pAT153²⁰ to simplify further analysis of the DNA.

To determine the relationship between these cloned DNAs, heteroduplexes between all three size classes of insert were

Thus, the two major size classes of cloned circular *copia* DNA molecule seem to comprise intact *copia* elements circularized at the extreme ends of the element with or without the deletion of one direct repeat sequence. This result is very similar to that observed in cloned circular retroviral proviruses which also possess either one or two direct repeat sequences (usually termed long terminal repeats^{21,22}). The latter configuration is labile in bacteriophage vectors, with loss of one direct repeat sequence sometimes occurring by recombination during propagation²³. To prove that the cloned *copia* species containing one direct repeat were not derived from such artefacts, a sample of uncloned total *Drosophila* circular DNA was digested with three restriction enzymes (*Hind*III, *Hinc*II and *Pvu*II) that easily distinguish between these two species. The *copia*-specific products of these digestions were identified by Southern filter hybridization. Fragments derived from molecules containing one direct repeat (0.9 kb) and two direct repeats (1.2 kb) in *Hinc*II/*Pvu*II double digests of uncloned *copia* circles were observed (Fig. 6, lanes c, e), showing that both types of molecule exist in *Drosophila* cultured cells.

Restriction fragments characteristic of 4.2-kb circles were not detected in digests of uncloned circular DNA. This suggests that

either these elements are derived from a cloning artefact or they constitute a small minority of the circular *copia* species. We favour the latter interpretation because deletions associated with prokaryotic transposons^{2,3} and in circular retrovirus proviruses^{24,25} have often been observed previously.

Origin of circular *copia* elements

Circular *copia* elements might be derived in several ways. First, they may be self-replicating plasmids. We think that this is unlikely because of their low abundance. Second, they may be synthesized by reverse transcription of *copia* RNA. Such RNA could be derived either from infecting '*copia* retroviruses' or possibly from the full-length intracellular *copia* RNA which is abundant in cultured *Drosophila* cells⁶. These hypotheses are supported by evidence of reverse transcriptase activity and the reported presence of retrovirus-like particles in *Drosophila* cells²⁶. However, we favour a third interpretation, that they are derived from the relatively infrequent excision of genomic *copia* elements.

Excision of genomic *copia* by homologous recombination between the terminal direct repeats would generate the observed 4.7-kb circles possessing one direct repeat. Such excision would leave single copies of the direct repeats behind in the *Drosophila* chromosomes. These single copies were not detected in embryonic cells¹³ but cultured cells, which possess approximately fourfold more *copias* per haploid genome, were not probed for such sequences. We therefore cannot exclude the possibility that the 4.7-kb molecules are derived from such a mechanism. Homologous recombination between direct repeats could not yield 5.0-kb molecules containing two repeat sequences, although recombination between the flanking host 5-bp repeats (Fig. 1) would create such species. However, if such a mechanism is implicated, there must be greater control of excision because any given pair of 5-bp sequences occurs about once in every 1,000 bp in the *Drosophila* genome. If the excision of 5.0-kb molecules is based on homologous recombination, one copy of the variable flanking 5-bp repeats might be present between the fused direct repeats in 5.0-kb circles. Alternatively, if the 5.0-kb circles are derived by reverse transcription, it is likely that all these molecules possess an invariant sequence at the junction between the two direct repeat sequences. The sequence of cloned 5.0-kb molecules in this region is now being determined to test these predictions.

Eukaryotic transposable elements and retroviruses

The experiments described here indicate that eukaryotic transposable elements and retrovirus proviruses share close structural similarities. The isolation of circular *copia* elements whose structures are closely analogous to circular proviruses extends these common properties. Such similarities could be a result of either a common ancestry or convergent evolution. Temin²⁷ has pointed out, however, that convergent evolution would be unlikely to produce such close structural similarity and such a high degree of sequence homology at the ends of these elements. It therefore seems likely that these sequences are evolutionarily related.

The degree of relationship between present day transposable elements and retrovirus proviruses is difficult to estimate. If these species are closely related it is possible that retroviruses can transpose without using RNA intermediates; there is no evidence to support or discredit this hypothesis. It is similarly possible that all eukaryotic transposable elements are retroviruses, although this seems highly unlikely in the case of yeast transposable elements. Furthermore, the presence of transposons in prokaryotes suggests that transposable genetic elements are more widespread than retroviruses and probably predate them evolutionarily.

We therefore favour the hypothesis that a broad spectrum of related species exists in eukaryotes between the transposable elements of yeast and the non-defective retroviruses of higher

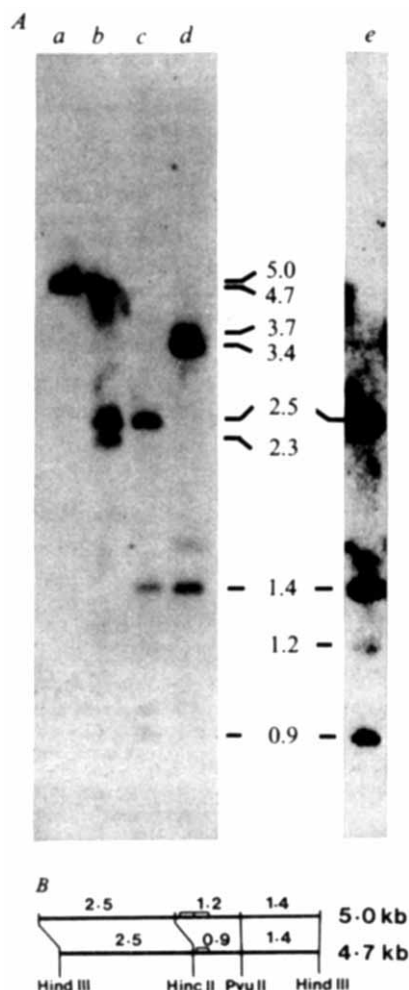


Fig. 6 Detection of circular *copia* molecules containing either one or two direct repeats in cultured cells. Samples of uncloned circular DNA from cultured cells were digested with restriction endonucleases and analysed for *copia*-specific sequences. **A**, lane a, *Hind*III plus *Msp*I; lane b, *Hind*III plus *Msp*I and *Hinc*II; lane c, *Hind*III plus *Msp*I, *Hinc*II and *Pvu*II; lane d, *Hind*III plus *Msp*I and *Pvu*II. Lane e is a longer exposure of lane c to reveal the 1.2-kb fragment characteristic of 5.0-kb circles. The sizes of *copia*-specific fragments were derived from comparison with parallel marker DNA fragments. **B**, the restriction maps of 5.0-kb and 4.7-kb insert DNAs for the enzymes used in these digests are shown. *Msp*I does not digest either the uncloned or cloned *copia* DNAs but aids complete digestion in preparations containing uncloned *copia* circles for reasons that are unclear. The location of the direct repeats is indicated (□).

vertebrates which usually, if not always, transpose via RNA intermediates. All these species may, however, still share common intermediates, such as circular DNAs, and all may use similar cellular enzymes in their integration. We are now investigating whether *copia* circles can integrate into the *Drosophila* chromosomes to create normal genomic *copia* elements. Of course, if circular *copia* molecules are derived by

excision of genomic material and if these circles can integrate in this manner, then they must be potential transposition intermediates.

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Transformation of rat cells by an altered polyoma virus genome expressing only the middle-T protein

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A modified polyoma virus genome has been constructed which can encode the middle-T protein, but not the large-T or small-T proteins. This was achieved, starting with the full length viral DNA inserted into a plasmid vector, by replacing a small genomic restriction fragment spanning the middle-T intervening sequence with the equivalent fragment from a cloned partial cDNA copy of the middle-T protein mRNA. Transfection of the modified viral DNA into cultured rat cells efficiently induced the formation of transformed cell foci which gave rise to cell lines that grew as tumours after injection into Fisher rats. The only viral early-region antigen synthesized by the cell lines was the middle-T protein. Expression of the middle-T protein is therefore sufficient to establish and maintain a transformed state. The viral mRNA produced by two of the transformed cell lines was structurally indistinguishable from the normal middle-T mRNA found in productively infected cells, suggesting that RNA splicing is not an essential step in the biogenesis of this messenger.

THE early region of polyoma virus DNA encodes at least three polypeptides, the large-T, middle-T and small-T proteins (refs 1–4; reviewed in ref. 5). Conclusive evaluation of the individual roles of these proteins in virally induced oncogenic transformation has been difficult because the DNA sequences^{6,7} which encode them overlap extensively (Fig. 1). It is known that expression of the large-T protein is insufficient to transform cells^{8,9}, and that the 3'-terminal half of the early region, which encodes only C-terminal sequences of large-T protein, is not required to maintain^{10,11} or to establish^{12–15} the transformed state. Attention has therefore focused on the 5'-terminal half of the early region, which encodes the small-T and middle-T proteins as well as the N-terminal portion of the large-T protein. As all previously reported mutations within this region of the genome affect at least two of the three T-proteins^{9,16–19}, rigorous assignment of the resulting phenotype to a change in any individual gene product has not been possible. We now report the construction of an altered polyoma virus early region which encodes exclusively the middle-T protein, and describe the mode and consequences of the expression of this DNA when it is introduced into cultured rat cells.

Construction of a viral early region encoding only middle-T protein

We constructed a modified viral early region encoding exclusively the middle-T protein by specific deletion of the middle-T protein intervening sequence (see Figs 1 and 2). We achieved this, starting with a recombinant plasmid containing full-length wild-type viral DNA (p53.A6.6), by replacing a small genomic restriction fragment spanning the small-T and middle-T protein intervening sequences with the corresponding shorter fragment from a cloned partial cDNA copy²⁰ of the middle-T protein mRNA (Fig. 2). From the structures of the Py early-region mRNAs (ref. 20; Fig. 1) we predicted that the plasmid produced, pPyMT1, would encode only middle-T protein. The polyoma virus early-region mRNAs are generated by the differential splicing of a common precursor. This involves the ligation of two splice donors (at nucleotides 409 and 746) to two splice acceptors (at nucleotides 795 and 809) in three of the four possible combinations (Fig. 1). The mature mRNA for middle-T protein (spliced from 746 to 809) lacks the acceptor (at 795) used in large-T and small-T mRNAs. Moreover, as the highly

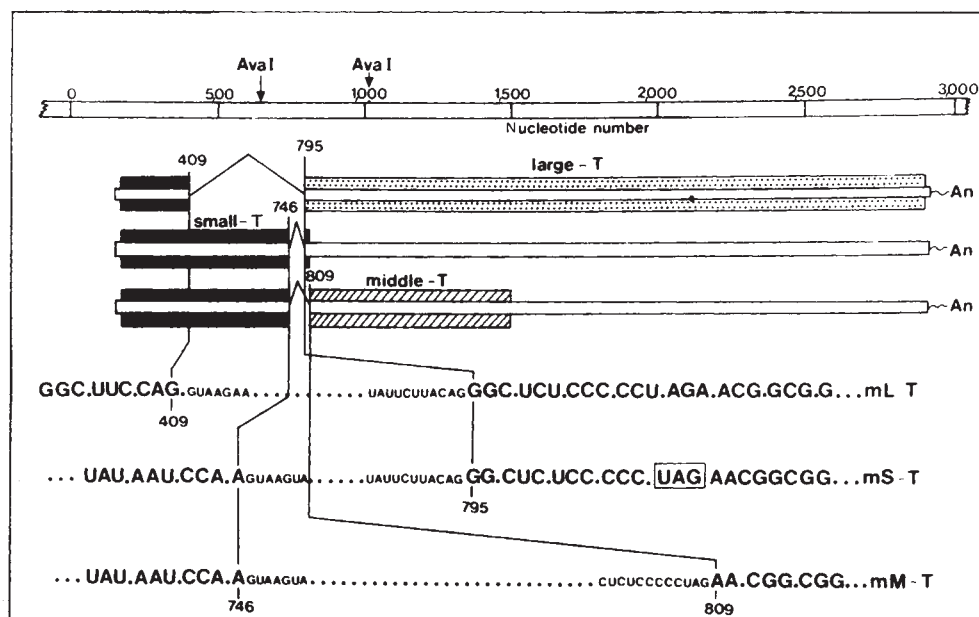


Fig. 1 The spliced structures of the principal Py early-region mRNAs. At the top of the figure is a linearized map of the Py early region, with conventional nucleotide numbers⁷ indicated. The three differentially spliced principal mRNAs²⁰ are aligned with this map. The coding regions of the mRNAs are boxed; solid boxes, dotted boxes and hatched boxes refer to reading frames (as defined in ref. 7) 1, 2 and 3 respectively. The nucleotide sequences across the splice joints²⁰ are shown in the bottom half of the figure.

conserved consensus sequences at splice donor sites lie largely within the intervening sequences^{21,22}, the splice donor at nucleotide 746 in the middle-T mRNA should also be inactive. The only potential splicing signal remaining in this mRNA would be the donor (at nucleotide 409) used in the large-T mRNA splice. The primary transcript of the viral early region in pPyMT1 (compare with sequence in Fig. 1) could therefore not be spliced to generate mRNAs encoding normal large- or small-T proteins, but it would encode the middle-T protein. A novel splice could perhaps join the large-T mRNA donor to an otherwise unused acceptor, but we considered this possibility to be improbable, and indeed such splices did not occur.

Transformation of rat cells with pPyMT1

We tested the biological activity of the viral genome in pPyMT1 by transfecting Fisher rat cells (F2408 cell line)²³, using the calcium phosphate technique²⁴, and quantifying dense foci overgrowing the normal monolayer which appeared after 2–3 weeks of incubation in the presence of 5% fetal calf serum (FCS). We used as a control the plasmid p53.A6.6, the parent of pPyMT1, which contains a normal polyoma virus genome (Fig. 2). In repeated assays, the transformation efficiency of pPyMT1 was 20–45% that of the control plasmid when non-saturating amounts of DNA were used (Table 1). No foci appeared when cells were transfected with carrier DNA alone, or with the plasmid vector and carrier DNA. Twenty foci induced by pPyMT1 and six induced by p53.A6.6 were picked and grown into cell lines for further analysis.

All the transformed cell lines were initially screened for the presence of viral T-antigens by indirect immunofluorescence using a serum from a Py tumour-bearing rat. Of the six control cell lines transformed by the unmodified parental plasmid, five showed the strong nuclear fluorescence pattern characteristic of the polyoma virus large-T antigen. None of the 20 cell lines derived from pPyMT1 transfections displayed such fluorescence, although many showed a weak specific immunofluorescence which appeared to be distributed throughout the cell.

Properties of rat cell lines transformed by pPyMT1

Eight pPyMT1 and two control lines were selected at random for more detailed study. The properties measured (Table 2) were saturation density, ability to form foci when grown with untransformed rat cells, ability to grow in semi-solid medium

and morphology at low- and high-cell densities (Fig. 3). All 10 cell lines displayed, by these criteria, the usual range of phenotypes characteristic of polyoma virus-transformed rat cells. Those transformed by pPyMT1 could not be distinguished from the control cell lines. We conclude that from all of the parameters tested that the lines derived from pPyMT1-induced foci are transformed. We do not know whether differences between these cell lines and those transformed by genomic viral DNA might be demonstrable by measurement of other parameters of the transformed state. In particular, we have noted that many, but not all, of the pPyMT1-transformants grew poorly on plastic when seeded at very low cell densities in 5% FCS. We have also measured the ability of four pPyMT1-transformed (1.6, 1.7, 2.4 and 2.8) and two control (4.1 and 4.6)

Table 1 Transformation of F2408 rat fibroblasts by pPyMT1 DNA and control plasmid DNA

Expt no.	Plasmid	Amount (μg)	No. of foci	Foci per μg viral DNA	Ratio pPyMT1/p53.A6.6	
I	pPyMT1	0	0	—	0.40	
		0.1	59	1,000		
		1.0	137	232		
	p53.A6.6	0	0	—		
		0.1	149	2,530		
		1.0	409	693		
II	pPyMT1	0.05	100	3,930	0.45	
		0.05	132			
		0.05	248			
	p53.A6.6	0.05	275	8,810		
		0.05	275			
		0.05	275			
III	pPyMT1	0	0	—	0.20	
		0.2	15	127		
		1	82	139		
	p53.A6.6	5	181	61		0.28
		0	0	—		
		0.2	76	644		
		1	294	498		
		5	> 500	—		
		0	0	—		
		0.1	77	1,305		
IV	pPyMT1	1.0	324	549	0.20	
		5.0	361	122		
		0	0	—		
		0.1	375	6,360		
	p53.A6.6	1.0	> 500	—		
		5.0	> 500	—		
		0	0	—		
		0.1	> 500	—		

Subconfluent monolayers of cells (90 mm cultures) were transfected with the indicated quantities of plasmid DNAs. Dense foci were counted 14–21 days later. In experiment II, the cells were trypsinized and divided among four dishes 24 h after transfection.

Table 2 Properties of pPyMT1- and control plasmid-transformed cell lines

Cell line	% Efficiency of focus formation*	% Efficiency of growth in agar†	Saturation density‡ (cells per $\text{cm}^2 \times 10^{-6}$)
pPyMT1			
1.2	0.4, 2.2	4.4, 10	>0.99
1.6	2.7, 3.2	6.2, 96	>1.4
1.7	3.7, 93	5.4, 60	>1.0
1.8	1.0, 5.0	3.3, 1.2	>1.1
2.4	2.3, 1.6	0.8, 1.6	>1.3
2.6	1.0, 2.4	0.8, 0.9	>1.1
2.8	1.4, 12.8	2.5, 0.9	>0.97
2.9	1.4, 4.4	2.5, 0.9	>0.83
p53.A6.6			
4.1	3.0, 4.7	1.7, <0.02	>0.70
4.6	1.7, 4.8	1.6, 20	>1.20
F2408 rat cells	none	<0.01	0.28

* Transformed cells (5×10^2 and 5×10^3) were plated in separate experiments with either 2×10^5 (first number) or 2×10^4 (second number) F2408 rat cells on 50-mm dishes. Foci were counted after 14 days of growth in Dulbecco's minimal essential medium (DMEM) containing 5% FCS.

† Transformed cells (5×10^2 and 5×10^3) were grown in DMEM, 5% FCS, 0.3% (w/v) agar. Microscopically visible colonies were counted after 14 days. Results of two independent assays are listed, done after extensive passage of the cell lines in culture (first number) or shortly after the original foci had become established as cell lines (second number).

‡ Cells (5×10^5) were plated on 50-mm dishes and grown in DMEM/5% FCS, with medium changes every 24 h after the third day. The control F2408 cultures reached the indicated density at day 4 and increased no further. All transformed lines continued to divide until the cells came away from the plastic; thus the numbers listed are minima.

cell lines to grow as tumours after injection into Fisher rats. Tumours were produced within 3 weeks of subcutaneous injection of 4×10^5 cells in all six cases; lines 1.6 and 2.4 produced tumours when only 4×10^4 cells were injected.

Viral proteins synthesized by pPyMT1-transformed cell lines

The prediction that the viral early region in plasmid pPyMT1 would encode only the middle-T protein was confirmed by analysis of the viral proteins synthesized by the transformed cell lines. A randomly selected set of cloned cell lines transformed by p53.A6.6 or pPyMT1 were labelled with ^{35}S -methionine and the proteins precipitable by polyoma virus anti-T serum were fractionated on SDS-polyacrylamide gels (Fig. 4). In eight of nine pPyMT1 cell lines tested, the only specifically immunoprecipitated protein detected co-migrated with the normal viral middle-T protein synthesized in mouse or rat cells infected with polyoma virus. One line, 2.9, additionally produced a smaller polypeptide which is probably a truncated middle-T protein. The amount of middle-T protein produced was within the usual range obtained with Py-transformed rat cells. No large-T, truncated large-T, or small-T proteins were found. These conclusions were strengthened by analysis of the viral mRNA present in the cell lines, as discussed below. Two control lines transformed by the control plasmid p53.A6.6 were similarly analysed. Both synthesized middle-T and small-T proteins. One (4.1) also produced a full-length large-T polypeptide, while the other (line 4.6, which was T-antigen negative by immunofluorescence) synthesized a shorter polypeptide which was probably a truncated large-T molecule, like those frequently found in other polyoma virus-transformed rat cell lines^{10,11}.

Viral mRNAs synthesized by pPyMT1-transformed cell lines

We investigated the structure of the viral mRNAs in several transformed lines for two reasons: first, to demonstrate unambiguously that the pPyMT1 transcripts were not spliced to produce mRNAs which could encode viral proteins other than middle-T; and second to know how the transformed cell lines generated stable cytoplasmic mRNA encoding middle-T

protein from the modified genome in pPyMT1. Previous reports²⁵⁻²⁷ had suggested that RNA splicing is an obligatory step in the expression of genes which normally contain intervening sequences. We therefore thought it possible that the transformed cell lines always contained viral genomes integrated such that the early region and a host intervening sequence could be co-transcribed. However, because several genes in higher eukaryotes are known to express unspliced transcripts as stable cytoplasmic mRNAs²⁸⁻³⁰, a direct mode of expression could not be discounted.

The S_1 -gel mapping experiments shown in Fig. 5 (see legend for details) clearly established that, in three of the four pPyMT1-transformed lines examined, the principal mRNAs had 5' termini mapping in the same position (around nucleotide 150; 73 map units) as those of productively infected cells or other cell lines transformed by genomic viral DNA^{11,31}, and that these mRNAs were colinear with the pPyMT1 DNA sequence throughout the region encoding the middle-T protein. This demonstrated that none of the usual splicing events had occurred. Three of the transformed lines also produced variable amounts of a transcript beginning somewhere in vector or host DNA sequence 5' to the early region, which was also unspliced. In other experiments not presented here, we used a 3'-labelled single-stranded DNA probe to detect specifically splices originating from the donor used in the large-T mRNA (see Fig. 1); control, but none of the pPyMT1-induced, cell lines used this

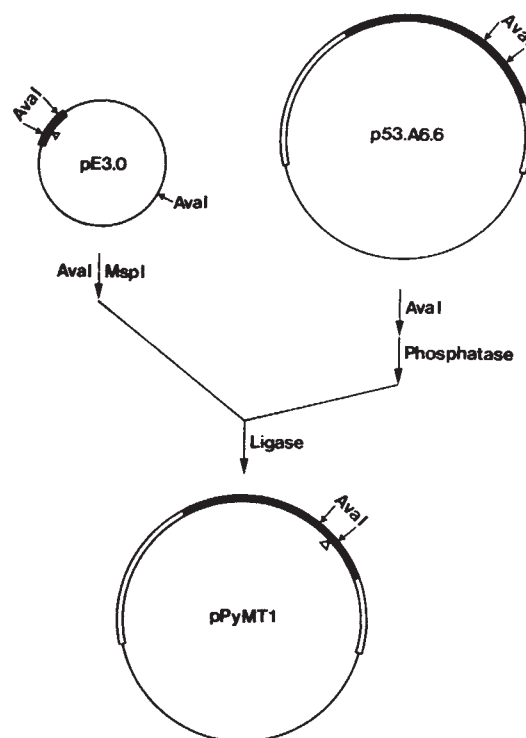


Fig. 2 Construction of a modified polyoma virus early region encoding only the middle-T protein. Plasmid pE3.0²⁰ consists of a partial cDNA copy of the middle-T protein mRNA (solid bar) inserted at the *Pst*I site of plasmid pAT153⁴⁵. The absence of the intervening sequence is indicated by Δ . Plasmid p53.A6.6 consists of a complete wild-type Py genome inserted via the unique *Bam*HI site into a derivative of pAT153 in which the plasmid *Ava*I site had been removed by cleavage with *Ava*I, S_1 nuclease treatment and recyclicalization. The Py early region is shown as a solid bar and other Py sequences as open bars. Plasmid pE3.0 was digested with *Ava*I + *Msp*I. Plasmid p53.A6.6 digested with *Ava*I was treated with calf intestine alkaline phosphatase⁴⁶. The enzyme was inactivated by heating at 70 °C for 1 h, and the DNA used for ligation without further treatment. Equimolar amounts of the two plasmid digests were ligated at 25 $\mu\text{g ml}^{-1}$ p53.A6.6. After transfection into *E. coli* HB101, ampicillin-resistant colonies were picked and plasmid DNAs prepared⁴⁷. Recombinants were analysed for the substitution of the Py genomic *Ava*I fragment by the pE3.0 cDNA *Ava*I fragment using restriction endonucleases *Ava*I, *Ava*II, *Hin*II and *Msp*I. One recombinant with the appropriate restriction map, pPyMT1, was chosen and large quantities of its DNA prepared⁴⁷.

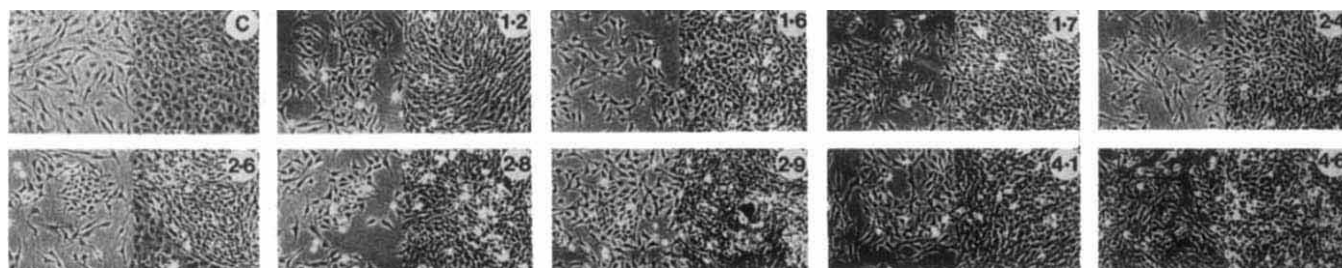


Fig. 3 Phase-contrast photomicrographs of pPyMT1- and p53.A6.6-transformed rat cell lines. Lines 1.2, 1.6, 1.7, 2.4, 2.6, 2.7 and 2.8 are pPyMT1 induced, 4.1 and 4.6 are p53.A6.6. 'C' is untransformed F2408 rat cells. Cells (5×10^5) were plated on 50-mm dishes and incubated at 37 °C with DMEM containing 5% FCS for either 1 (left panels) or 4 (right panels) days.

splicing donor. Therefore, the only known early-region viral polypeptide which could be made from the mRNAs in pPyMT1-transformed cell lines is the middle-T protein.

The data shown in Fig. 5 also established that, at least in the two pPyMT1 lines mapped in detail (lines 2.4 and 2.8), viral mRNAs were produced which were colinear with the modified viral DNA sequence for the entire extent of the early region and had polyadenylated 3' ends mapping within viral sequence at the normal position. This strongly suggested, for reasons to be discussed below, that RNA splicing was not involved in the biogenesis of these messengers. Maps of the viral mRNAs produced by pPyMT1-transformed lines and by one p53.A6.6 line (4.6) are shown in Fig. 6. The third pPyMT1 line (1.2) examined in detail had mRNAs with 3' ends of colinear viral sequence which mapped within that portion of the early region which, in genomic DNA, uniquely encodes the C-terminal part of large-T protein. This has often been found in other Py-transformed rodent cell lines, and indeed is also the case with the one control line analysed here (line 4.6, see Fig. 6). It usually indicates the position of a virus-host integration junction^{10,11}. As such integration events remove the principal viral polyadenylation site, cell lines like 1.2 and 4.6 have often been found to produce relatively large amounts of mRNA terminating at the alternative viral polyadenylation site at 99 map units^{10,20}, as illustrated in Fig. 6. One pPyMT1 cell line (2.8; see Fig. 6) also produced a continuous transcript of the L-DNA strand of the late region, extending from a 5' end at an undetermined point in

the flanking vector or host DNA sequence to a polyadenylated 3' end mapping at the same position as that of late-region mRNAs produced in lytically infected cells.

The middle-T mRNAs synthesized by the pPyMT1-transformed cell lines 2.4 and 2.8, are identical in structure to the middle-T mRNAs produced during productive infection (Fig. 6). The mRNAs in the transformed lines, however, have not been spliced between their 5' ends and polyadenylated 3' termini. This strongly suggests that RNA splicing does not occur during the biogenesis of these messengers. We cannot rigorously exclude the alternative possibility, that the mRNAs derive from longer precursors which had been spliced and then subsequently processed to generate the 5' or 3' ends of the mature species, but we consider processing at the 5' end unlikely, because the ends detected are indistinguishable from those generated by normal use of the viral early promoter³¹; it is firmly established in several other systems^{32,33} that mRNA 5' ends correspond to transcriptional initiation points. We believe that cleavage and 3' polyadenylation after downstream splicing is equally improbable because polyadenylation has been shown to precede splicing during processing of Ad2 and simian virus 40 (SV40) RNAs^{34,35}. The efficiency with which the transformed lines produce cytoplasmic mRNAs may, however, be slightly impaired by the absence of splicing, as we have noted above that the transformation efficiency of pPyMT1 DNA is two to three times lower than that of the parental plasmid DNA. The level of expression obtained from integrated Py DNA has been shown to

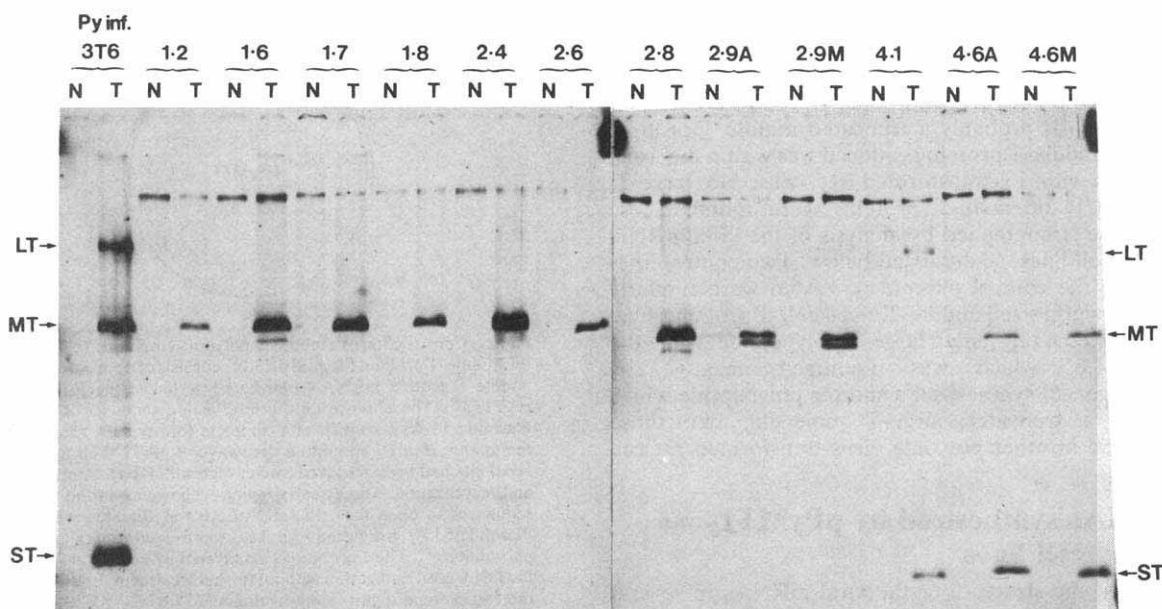


Fig. 4 Viral proteins synthesized by rat cells transformed by modified plasmid pPyMT1 (cell lines 1.2, 1.6, 1.7, 1.8, 2.4, 2.6, 2.8, 2.9A [after cloning in soft agar] and 2.9M, which had been cloned in Methocel) or by control plasmid p53.A6.6 (cell lines 4.1 and 4.6). Cultures grown in 50-mm plates were labelled with ³⁵S-methionine (0.5 mCi per plate) for 3 h. Proteins were extracted and precipitated with Py anti-T protein serum (lanes T, immune serum; lanes N, non-immune serum) as described previously¹; they were then fractionated on 15% SDS-polyacrylamide gels, using the viral proteins precipitated from Py-infected mouse cells as markers (M).

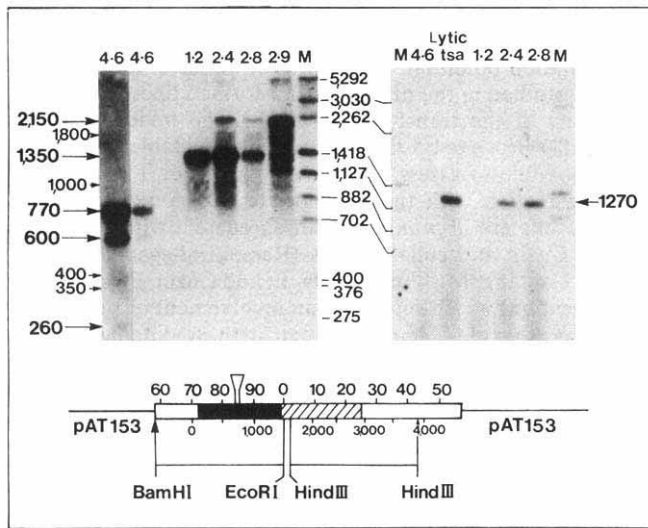


Fig. 5 Characterization of the viral mRNA in pPyMT1-transformed (1.2, 2.4, 2.8 and 2.9) and a p53.A6.6-transformed (4.6) cell lines by alkaline agarose gel electrophoresis of nuclease S_1 -resistant hybrids. Experimental procedures were previously described⁴⁸ in detail. The diagram illustrates the structure of the Py DNA insert in the plasmids, with the middle-T protein-coding region in black, the remainder of the early region hatched and the restriction fragments used in the S_1 -mapping experiments indicated. The position of the deletion in pPyMT1 is also shown (V). Left panel: S_1 -resistant DNA from 100 μ g of total cytoplasmic RNA from the indicated cell lines, hybridized to the unlabelled homologous DNA fragments extending from a BamHI to an EcoRI (58.0–100 map units; nucleotides 4,632–1,565; see diagram) site in plasmids pPyMT1 or p53.A6.6. After electrophoresis, the S_1 -resistant DNA products were transferred to nitrocellulose and visualized by annealing to nick-translated viral DNA. The lane on the left is from a longer exposure of the same gel (on this exposure, the indicated 350-nucleotide product was visible with the pPyMT1 transformants). Lane M shows Py restriction fragments run as size markers. The calculated lengths of S_1 -resistant products are indicated to the left. Larger numbers refer to principal products. Smaller numbers refer to products resulting from S_1 cleavage within DNA-RNA hybrids at 93 map units, a phenomenon always found in S_1 mapping of Py early-region mRNAs which is probably a methodological artefact²⁰. Product assignments are: lane/line 4.6: the 600- and 260-nucleotide products represent the alternative 5'-colinear RNA segments, as shown in Figs 1 and 6; the 770-nucleotide product represents the 3'-colinear RNA segments, extending from the splice acceptors at nucleotides 795 and 809 to the end of the restriction fragment at 1,565; the minor 400- and 350-nucleotide products derive from the 770 by S_1 cleavage at 93 map units (~nucleotide 1,200). In lanes/lines 1.2, 2.4 and 2.8, the 2,150-nucleotide product represents a continuous transcript extending from the BamHI site to the EcoRI of pPyMT1 DNA; the 1,800-nucleotide product is the result of cleavage of the 2,150 at 93 map units; the major 1,350-nucleotide product represents a continuous transcript extending from the normal 5'-end position of Py early-region mRNAs (nucleotide 150; ref. 31) to the EcoRI site in pPyMT1 DNA; the minor 1,000- and 350-nucleotide products represent cleavage of the 1,350 at 93 map units. Lane/line 2.9: pattern too complex for analysis. Right panel: polyadenylated cytoplasmic mRNA from the indicated transformed cell lines, or from mouse cells productively infected with Py thermolabile mutant *tsa*²⁰, annealed to the unlabelled Py HindIII fragment illustrated in the diagram below. The transfer was annealed to a ³²P-labelled probe⁴⁸ which detects only transcripts of the E-DNA strand, and therefore the 1,270-nucleotide product maps the 3'-colinear segments of early-region mRNAs extending from the HindIII site (1.8 map units; nucleotide 1,650) to the previously determined polyadenylation site at 25.8 map units (~nucleotide 2,930). No bands were detected with RNA from cell lines 4.6 and 1.2 because the 3' ends of viral sequence in these mRNAs lie before (1.2) or shortly after (4.6) the HindIII site.

synthesis and instead reflect the absence of the small-T protein (see below).

To investigate this alternative, we have begun experiments comparing nuclear RNA with cytoplasmic RNA in the pPyMT1-transformed cell lines. We are also studying the expression of pPyMT1 DNA soon after transfection into rat cells; preliminary results indicate that the amount of viral cytoplasmic RNA synthesized from the modified genome is similar to that produced by unmodified DNA 60 h after DNA transfection, but better quantification is required to resolve the issue.

The production of stable, unspliced, cytoplasmic middle-T mRNA from the modified genome in pPyMT1 does not necessarily conflict with previous results^{25–27}. The conclusion that RNA splicing is obligatory could not be generalized from the few cases examined in these earlier experiments. Moreover, if instead of proposing that splicing in itself is essential, one hypothesizes that there is an RNA sequence, often but not always located within introns, which has a positive function in RNA maturation, all the available data could be accommodated. Given the pattern of differential splicing evolved by polyoma virus to produce three mRNAs from one precursor, it

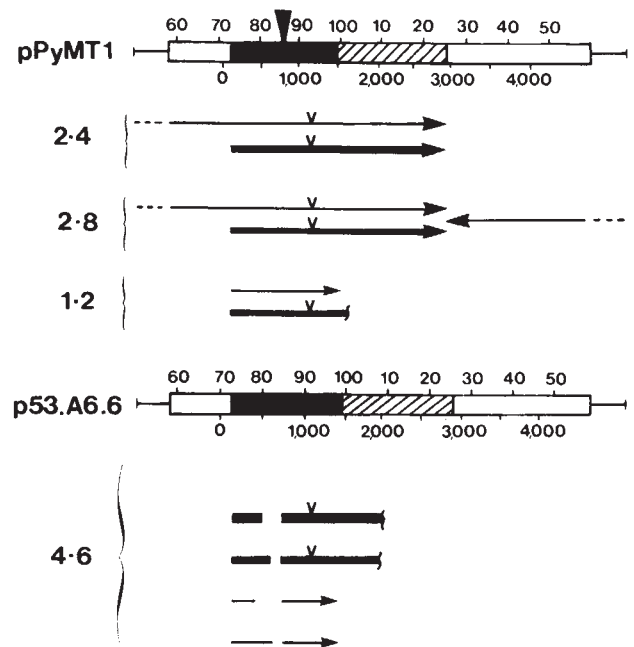


Fig. 6 Structures of Py mRNAs in pPyMT1- and p53.A6.6-transformed rat cell lines, as deduced from S_1 -gel mapping studies such as those illustrated in Fig. 5 and other experiments (not presented) using one- and two-dimensional gel fractionation. The mRNAs are aligned below line diagrams of the viral DNA inserts in the plasmids used, but note that we have not determined the structures of the viral DNA actually present in the transformed cell lines. The middle-T protein-coding regions are shaded, and the rest of the early regions are hatched. mRNAs are represented by thick and thin lines to suggest their relative abundancies. The 5' ends of readthrough transcripts have not been mapped and are therefore indicated by dotted lines. Arrow heads are polyadenylation sites. The S_1 -sensitive site at 93 map units is indicated by V. The 3' ends of viral sequence indicated by } probably represent the positions of virus-host joints, as frequently found in other Py-transformed cell lines¹⁰. The four cell lines all contained detectable amounts of mRNAs ending at the 99-map unit alternative polyadenylation site^{10,20}, but these are only illustrated for lines 1.2 and 4.6 where they comprised significant proportions of the viral messenger. Note that in cell line 4.6 there must be mRNAs with the two different small splices (see Fig. 1) because this line produces both small- and middle-T proteins, but these cannot be distinguished by agarose gel analysis.

vary considerably among transformed cell lines, although each seems to use the viral early promoter¹⁰. This variation is probably a chromosomal position effect; similar observations have been made in avian sarcoma virus-transformed mammalian cell lines³⁶. Thus, we may have selected among the pPyMT1 transformants a subset of integrated genomes which are transcribed at a particularly high level to compensate for a defect in subsequent processing. Alternatively, the reduced trans₀₁₃₆ formation efficiency of pPyMT1 may have little to do with RNA₀₁₄₅

would not be surprising if such a sequence occurred somewhere outside the 63 nucleotides comprising the middle-T protein intervening sequence. This hypothesis would also better explain the expression of genes such as those for adenovirus protein IX²⁹ and interferons³⁰ which lack introns, or the abundant production of unspliced late SV40 19S mRNA²⁸ in certain situations. The effect of deletion mutations interfering with the splicing of the SV40 small-T protein mRNA has also been investigated³⁷; unspliced mRNAs were usually not expressed. It is possible, however, that most of the deletions caused preferential splicing of the precursor to yield large-T, rather than unspliced, mRNAs. Indeed, one deletion (dl 884) removing the small-T mRNA splice donor appeared to synthesize a truncated small-T protein, perhaps by translation of an unspliced messenger³⁷.

Roles of polyoma virus early proteins in transformation

Our results strongly suggest that expression of the polyoma virus middle-T protein is sufficient to establish and maintain a transformed state. What of the other two viral early proteins? Viable deletion mutants (the hr-t mutants) have been isolated⁹ which synthesize normal large-T protein but no detectable small- or middle-T proteins^{4,38,39}. These mutants do not transform cells^{8,9}. Moreover, the integration of such DNA into the rat cell genome can result in cell lines which express the large-T protein but are phenotypically normal⁸. Deletion mutants lacking DNA encoding the C-terminus of the middle-T protein, but which encode normal small-T protein, are severely impaired in transforming activity⁴⁰, as are certain internal deletions mapping within the middle-T/large-T overlap region¹⁷⁻¹⁹ and a point mutant which causes premature termination of only the middle-T protein (D. Templeton and W. Eckhart, manuscript in preparation). These observations are consistent with our demonstration of the critical role of middle-T protein in transformation; they also suggest that expression of the small-T protein alone cannot transform cells. It is therefore clear that of the three early polypeptides, only individual expression of the middle-T protein can induce the dramatic changes in cell regulation identified as viral transformation. We found, however, that pPyMT1 DNA has a slightly reduced transformation efficiency, which may reflect either an impairment in middle-T gene expression or the absence of an additional gene product. It will be interesting to construct plasmids containing two modified early regions which

independently encode middle-T and one of the other T proteins and to determine whether any of these have a fully restored transformation potential. Furthermore, we have not yet systematically studied in the pPyMT1-transformed lines all relevant parameters of the transformed state. If the middle-T transformed lines prove to be different in some selectable property, it will be possible to assess the roles of the other T proteins by supertransfection with the appropriate cDNA recombinants.

Our results are in *prima facie* disagreement with the recent work of Cuzin and collaborators (Rassoulzadegan, M., Gaudray, P., Canning, M., Trejo-Avila, L. and Cuzin, F., submitted for publication) which suggested an involvement of the amino-terminal portion of the large-T protein in the maintenance of the transformed state. The disagreement may reflect differences in the way in which the transformants were obtained, although preliminary results indicate that pPyMT1 DNA does transform FR3T3 rat cells in the conditions used by these workers (F. Cuzin, personal communication). Collaborative experiments in progress between the two laboratories using the plasmid DNA described here should resolve this issue.

The mechanism whereby middle-T protein causes transformation is unknown. Of the three polyoma virus T proteins, a unique property of the middle-T is its association with the plasma membrane^{16,38}, although efforts to detect it on the cell surface have been unsuccessful³⁹. Several groups have also found a protein kinase activity associated with the Py middle-T protein which results, *in vitro*, in the phosphorylation of a tyrosine residue in the middle-T protein itself⁴¹⁻⁴⁴. We tested four pPyMT1-transformed (1.2, 2.4, 2.6 and 2.8) and one p53.A6.6-transformed (4.6) cell lines for this kinase activity: they were all positive (data not presented), demonstrating that no viral protein other than the middle-T is required. However, it is not proved that the kinase is an activity of the middle-T protein rather than that of a bound host enzyme. Introduction of the modified viral early region from pPyMT1 into a bacterial expression vector to obtain large amounts of the middle-T protein would therefore be important to produce a middle-T protein that could be used to assess directly whether it is a tyrosine-specific protein kinase and to assay other potential functions.

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Induction of 8-azaguanine resistance and sister chromatid exchange in human lymphocytes exposed to mitomycin C and X rays *in vitro*

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Indirect evidence implies that 8-azaguanine-resistant (AG^r) lymphocytes in human peripheral blood are mutants associated with the loss of the hypoxanthine-guanine phosphoribosyltransferase (HPRT) locus on the active X chromosome, the mutation frequency increasing linearly with age. AG^r variants are readily induced in lymphocytes exposed to mitomycin C in vitro, their incidence correlating with induced sister chromatid exchanges (SCEs). Although SCE events and the development of an AG^r phenotype may reflect a common type of DNA damage, mitomycin C-induced AG^r variants are not mutants but are suggested to be cells having a transcriptional block at the HPRT locus. AG^r variants are also readily induced by X rays in vitro, their incidence correlates closely with the incidence of aberrations induced in the X chromosome and they are considered to have a mutational origin.

A MAJOR requirement in attempts to measure the effects of environmental agents in inducing mutations in man is the development of systems to measure induced mutation frequency in readily accessible human somatic cells. In man, the enzyme hypoxanthine-guanine phosphoribosyltransferase (HPRT) (EC 2.4.2.8) salvages hypoxanthine and guanine by conversion to their respective nucleotides, but the presence of this enzyme, which is coded for by an X-linked gene, is not necessary for cell proliferation and is absent, or defective, in males with the Lesch-Nyhan syndrome¹⁻³. The enzyme also converts purine analogues such as 8-azaguanine (8-AG) or 6-thioguanine (6-TG) to their respective nucleotides and, as these are cytotoxic, their incorporation results in cell death^{4,5}.

Strauss and Albertini⁶ used the cytotoxicity of 6-TG to human blood lymphocytes possessing normal HPRT, to distinguish and select for viable variant cells resistant to 6-TG (TG^r). Autoradiography indicates that such cells undergo DNA synthesis and survive in the presence of normally toxic levels of 6-TG following their exposure to the mitogen phytohaemagglutinin (PHA). The frequency of TG^r cells in lymphocyte populations from normal individuals was $\sim 1.3 \times 10^{-4}$ at a 6-TG concentration of 2×10^{-4} M, whereas in cancer patients being treated with cytotoxic (and mutagenic) drugs, their frequency was increased, in some cases up to 20-fold.

Small lymphocytes have scanty cytoplasm, a low metabolism and poor conversion of purine bases into nucleotides⁷. However, exposure to PHA initiates *de novo* purine synthesis and activates the purine salvage pathways⁸. It seemed possible therefore that exposure of lymphocytes to mutagens *in vitro* might also yield variant ('mutant') cells that would undergo blast transformation and remain viable on exposure to PHA and toxic purine analogues, in contrast to normal cells. We show here that the spontaneous frequencies of human lymphocyte variants resistant to 8-AG (AG^r variants) increase with the age of the blood donor; that variant frequency is increased in a dose-dependent fashion following exposure of human lymphocytes *in vitro* to the alkylating agent mitomycin C (MMC) or to X rays; that, for MMC, their incidence is related to the incidence of induced sister chromatid exchanges (SCEs) and, for X rays, is correlated with the incidence of gross chromosomal aberrations.

Rationale

One of the preliminary experiments undertaken to develop the protocol outlined below was a comparison of the response of lymphocytes to 8-AG and 6-TG (Fig. 1). The spontaneous incidence of purine analogue-resistant cells (defined by their

ability to undergo transformation by PHA and proceed through to a DNA replication phase) decreased with increasing analogue concentration, reaching a plateau at concentrations of $\sim 2 \times 10^{-4}$ M. Factors favouring the use of selection in 6-TG rather than 8-AG^{9,16} were: differences in the stability of the two analogues^{9,10}, the higher affinity of 6-TG than 8-AG for the substrate HPRT^{11,12}, the influence of exogenous purines present in serum in reducing the selection pressure exerted by 8-AG, but not 6-TG^{10,13}, and the fact that many mammalian cell mutants retrieved following selection in 8-AG may retain almost normal levels of HPRT whereas virtually all mutants following selection in 6-TG are HPRT⁻ (refs 14, 15). Nevertheless, similar mutant expression times and rates of spontaneous and X ray-induced mutations to AG^r and TG^r have been reported for human diploid fibroblasts¹⁷. In our study we were strongly influenced by the fact that 6-TG is incorporated into DNA^{5,18} and that inhibition of DNA synthesis by excess thymidine protects against the toxicity of 6-TG, but not of 8-AG⁵. In contrast, 8-AG is incorporated into RNA^{5,19}, and inhibition of RNA synthesis protects against the killing effects of 8-AG, but not 6-TG. The short-term culture of lymphocytes *in vitro* is inappropriate for selecting for resistance to cell killing after DNA synthesis, because the cells have a limited proliferation potential *in vitro* and colony-forming assays are not possible. In view of this, and because PHA-induced lymphocyte blast transformation requires considerable RNA synthesis, we used 8-AG (2×10^{-4} M) rather than 6-TG in all the experiments reported here.

Incidence of AG^r lymphocytes in relation to donor age and sex

The incidence of AG^r lymphocytes was determined in blood samples from 26 healthy donors (14 male) aged 16–82 yr (Fig. 2). The variant frequency ranged from 0.80×10^{-4} to 3.99×10^{-4} and despite considerable scatter, there is, nevertheless, a clear age dependence: a similar scatter, but no obvious age dependence, was noted by Strauss and Albertini⁶. There was no evidence of any significant effect of sex ($t_{24} = 0.76$, $P > 0.10$) and Kendall's²⁰ coefficient of rank correlation for the pooled data was $y = 0.325 \pm 0.140$ ($t = 2.33$, $P = 0.02$), indicating a significant age effect. The slope of the fitted line of least squares gives $b = 0.0294 \pm 0.0099$, so that the AG^r variant frequency increases by $\sim 3 \pm 1$ cells per 10^6 cells per yr. Interestingly, the two highest yields in the <45-yr age group were from blood samples from cigarette smokers, but almost equivalent yields

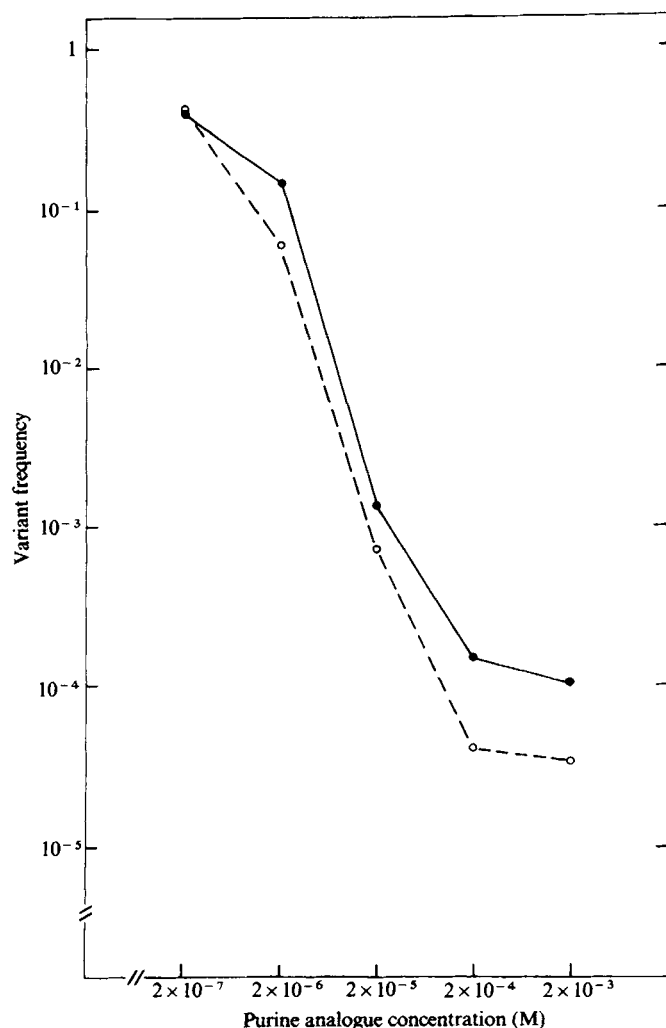


Fig. 1 The incidence of purine analogue-resistant variants in lymphocytes cultured in the presence of various concentrations of 8-azaguanine (●) or 6-thioguanine (○). In these and the other reported experiments, lymphocytes were separated by underlaying 3-ml aliquots of heparinized whole blood with 8 ml of Ficoll-Hypaque (10:5:2 of 12% Ficoll, 34% Hypaque and distilled water) and centrifuged for 15 min at 600g. The WBC layer was removed, washed twice with phosphate buffered saline and the cells resuspended in RPMI 1640 medium with 12.5% fetal calf serum, 1.8% PHA (Wellcome), 1% glutamine, 100 μ g streptomycin and 100 U penicillin. Duplicate sets of 5-ml cultures containing $2-3 \times 10^6$ lymphocytes were set up, one with and one without 8-AG or 6-TG, and incubated at 37 °C for 40 h. 3 H-thymidine at 1 μ Ci ml $^{-1}$ (specific activity 18 Ci mmol $^{-1}$) was added to all cultures 12 h before collection. The procedure then followed that described by Strauss and Albertini⁶, including repeated syringing of resuspended pellets in small volumes of methanol/acetic acid (5:1) fixative using a 25-gauge spinal needle fitted to a 1-ml syringe to give free nuclei. Suspended nuclei were dropped onto clean microscope slides, air-dried and stained with carbol fuchsin (3% in ethanol) before being dipped in L4 liquid emulsion and exposed in the dark at 4 °C for 72–96 h. As the work involved counting a considerable number of nuclei (up to 5×10^6 in some experiments), use was made of an automated computer image analysis system to count the total numbers of nuclei on the slides to be scored and the coded slides were then analysed by one observer by high-power light microscopy to obtain a count for the number of labelled nuclei. For all paired treatments the incidence of labelled nuclei was then determined as a labelling index and the variant frequency (Vf) was calculated as

$$Vf = \frac{\text{labelling index in the presence of AG}}{\text{labelling index in the absence of AG}}$$

were found in non-smokers and the limited numbers in our sample prevented any useful analysis of this parameter.

The incidence of 'spontaneous' and environmentally induced mutations in the somatic and germ-line cells of an individual would be expected to increase with increasing age and there is good evidence for an increased incidence of mutations transmitted to offspring in several studies on human populations^{21,22}.

Our results now provide evidence for an age-related increase in 'mutation' frequency in man's somatic cells. Ageing is associated with an increasing frequency of hypodiploid blood lymphocytes which in males consist largely of cells lacking the Y chromosome and in females of cells lacking an X chromosome, usually the inactive X^{23,24}. Ageing is also associated with smaller increases in the frequency of gross chromosomal structural aberrations (H.J.E., unpublished data), although aberration frequencies are of the order of 0.01 per cell whereas in ageing females more than 10% of the peripheral lymphocytes may be hypodiploid. Loss of an active X and consequent depletion of HPRT would result in the expression of an AG^r phenotype. As the incidence of AG^r lymphocytes is the same as between males and females, it seems more likely that a significant number of mutant AG^r cells may be deficient for functional HPRT due to deletion or structural change at the HPRT locus. The gene is located in the terminal band (q27) of long arm of the X chromosome²⁵, so that all deletions involving this arm will result in the loss of the HPRT locus. If breakage sites are distributed between chromosomes in proportion to their length, 1/40th of such sites would be in the X chromosome in a male cell and some 60% of these would be in the long arm (Xq). With a deletion incidence of ~0.01 per cell, a deletion of the terminal region of Xq should therefore occur at a frequency of $\sim 1.2 \times 10^{-4}$ per cell, a value comparable with the observed incidence of AG^r cells. In a study of G-banded chromosome preparations from individuals aged 60–90 yr we have identified a single Xq deletion in 2,131 cells—a frequency that again accords with the conclusion that the AG^r lymphocytes *in vivo* are probably cells with a deletion of Xq.

Induction of AG^r variants and SCEs following exposure to mitomycin C

Lymphocyte cultures were exposed to different concentrations of MMC for different time periods (see Table 1). Within each treatment time of 1, 5 and 10 h, the yield of MMC-induced AG^r variants increased with MMC concentration, each dose-response curve being of the form $y = \alpha + \beta d$, where y = variant frequency, α = control frequency of $8.38 \pm 0.37 (\times 10^{-4})$ and $d = [\text{MMC}]^{0.33}$. Least squares analysis gave values of β_1 , β_5 and β_{10} , respectively, of 0.83 ± 0.21 , 0.92 ± 0.15 and 1.06 ± 0.13 . Although the product of MMC concentration and exposure time would, for certain combinations, be equivalent between the three sets of data, the effective concentration of MMC decreases with time in culture such that for a given variant frequency, a quadrupling of exposure time is equivalent to a halving of the initial concentration of mutagen. The maximum increase in AG^r variant frequency is around 12-fold at the highest MMC concentrations, but significantly increased frequencies are evident following exposures to low doses, of the order of 10^{-8} M, over short time periods.

The data obtained in the parallel cultures on SCE incidence (Table 1) show the very much higher frequencies of induced SCEs relative to induced AG^r variants per cell, but the shapes of the dose-response curves for the two end points are very similar. Least-squares analysis of the SCE data fitted to $y = \alpha + \beta d$ gave a value of $\alpha = 7.63 \pm 0.19$ and the slopes of β_1 , β_5 and β_{10} were, respectively, 0.53 ± 0.03 , 0.60 ± 0.03 and 0.81 ± 0.06 .

The similarities in the kinetics of response to AG resistance and SCE induction are shown in Fig. 3 where each datum on AG^r variant frequency per cell has been multiplied by a factor of 7×10^3 and plotted against SCE frequency per cell. The resultant plot gives an excellent fit to a line of unit slope, indicating that for every induced AG^r variant in the cell population there are some 7,000 induced SCE events. If the AG resistance involves an alteration on a single X chromosome, because that chromosome represents some 2–3% of the total chromatin per male cell²⁶, then for every event on an X chromosome that would result in an AG^r cell there would be around 200 X-chromosome events that would yield SCEs. As SCE events may occur over almost the whole length of the chromosome, but

Table 1 Variant frequency and SCEs in human peripheral blood lymphocytes exposed to mitomycin C

Mitomycin C concentration	Exposure time (h)	+Azaguanine			-Azaguanine			Variant frequency ($\times 10^{-4}$)	SCEs per cell
		Labelled cells	Cells counted	Labelling index ($\times 10^{-3}$)	Labelled cells	Cells counted	Labelling index		
Control	1	20	229,492	0.087149	514	5,000	0.1028	8.48 $\pm$ 1.93	7.8 $\pm$ 0.31
10^{-7} M	22	22	248,372	0.088577	94	5,000	0.0188	47.12 $\pm$ 11.20	31.8 $\pm$ 1.11
10^{-6} M	10	10	108,928	0.091804	44	5,000	0.0088	104.32 $\pm$ 36.40	65.0 $\pm$ 1.94
10^{-5} M	—	—	—	—	—	—	—	—	—
Control	5	35	435,714	0.080328	469	5,000	0.0938	8.56 $\pm$ 1.50	7.4 $\pm$ 0.34
10^{-8} M	24	24	178,621	0.134363	223	5,000	0.0446	30.13 $\pm$ 6.47	19.6 $\pm$ 0.77
10^{-7} M	39	39	291,452	0.133813	126	5,000	0.0252	53.10 $\pm$ 9.73	38.5 $\pm$ 1.23
10^{-6} M	24	24	225,397	0.106479	50	5,000	0.0100	106.48 $\pm$ 26.30	70.2 $\pm$ 2.23
Control	10	52	627,603	0.082855	509	5,000	0.1018	8.14 $\pm$ 1.19	7.7 $\pm$ 0.33
10^{-9} M	126	126	866,525	0.145408	453	5,000	0.0906	16.05 $\pm$ 1.61	16.6 $\pm$ 0.82
10^{-8} M	86	86	481,292	0.178686	233	5,000	0.0466	38.34 $\pm$ 4.83	30.9 $\pm$ 1.43
10^{-7} M	84	84	576,284	0.145761	110	5,000	0.0220	66.26 $\pm$ 9.61	45.2 $\pm$ 1.46

The cells used were separated lymphocytes from 10–20-ml samples of blood from a single donor exposed to MMC at the time of culture initiation. They were washed after treatment and transferred to fresh medium containing PHA, with or without 8-AG, for 40 h at 37 °C and treated and processed as described in Fig. 1 legend. To determine SCE frequencies, a third set of cultures was set up following exposure to MMC, using complete medium containing 25 μ M bromodeoxyuridine and these were collected after 72 h and slides stained to give harlequin-stained chromosomes²⁸.

alterations at, or associated with, the HPRT locus to give an HPRT⁻ phenotype would be localized to only a small region, the numerical association between induced SCE events and AG^r variant induction is quite striking. This, however, does not imply a common mechanism for these two end points, but may simply reflect the fact that both types of event are consequences of the interaction of MMC with the cellular DNA.

Carrano *et al.*²⁷ have also reported a positive linear correlation between SCE and AG^r induction in Chinese hamster fibroblasts exposed to MMC: calculations on their data imply some 5×10^5 SCE events for each induced AG^r cell—a relative variant incidence some two orders of magnitude less than in the present human lymphocyte experiments. Studies on spontaneous mutation rates to AG^r in cultured human fibroblasts^{17,27}, and to TG^r in human lymphoblastoid cells²⁸, give values of 5×10^{-6} – 5×10^{-5} per cell generation, although both higher and lower values have been reported for other cell systems^{29,30}. The reported⁶ spontaneous frequency of TG^r lymphocyte variants of 1.3×10^{-4} and our values of 8×10^{-5} – 8×10^{-4} for AG^r variants seem too high to reflect mutational events at a single locus, but, as we have seen, they are consistent with the notion that many of these spontaneous variants lack part of an X chromosome.

Although the spontaneous AG^r variants may be a consequence of chromosomal aberration or loss, such changes cannot be responsible for AG^r cells induced following MMC treatment, because cells identified as AG^r are transformed by PHA and pass through the G₁ into the S phase, whereas normal

cells are blocked in G₁. However, the development of chromosomal aberrations following exposure to MMC occurs during the S phase and not before it³¹; moreover, chromosome loss could only occur at mitosis, long after cells are identified as AG^r cells. Similarly, induced variants are not base loss or substitution mutants at the HPRT locus following misreplication of damaged DNA at the S phase, although such changes might occur following DNA misrepair in G₁. What, then are these variants?

The true nature of the induced AG^r variants can only be clearly ascertained by studying their progeny: unfortunately, PHA-transformed lymphocytes normally have a limited lifespan in culture, so that this is not a practical proposition. However, there is a wide phenotypic diversity among purine analogue-resistant mammalian cells embracing variants with different mutations at the HPRT structural gene, or in associated regulatory loci³², as well as unstable cells with high reversion rates and considered to be 'non-mutant'³³. If the MMC-induced AG^r lymphocytes are not true mutants, an appealing possibility is that they represent cells in which MMC alkylation had occurred at sites on the HPRT locus where the large MMC-guanine adduct prevented transcription of the locus to give functional HPRT—and hence allowed the cells to survive in the presence of AG. Some of these hypothesized transcriptional blocks could well lead to mutation following replication, but this is not necessarily so. The transcriptional block hypothesis is not easy to test, but one approach would be to examine the response to AG^r induction in these G₁ cells by other mutagens.

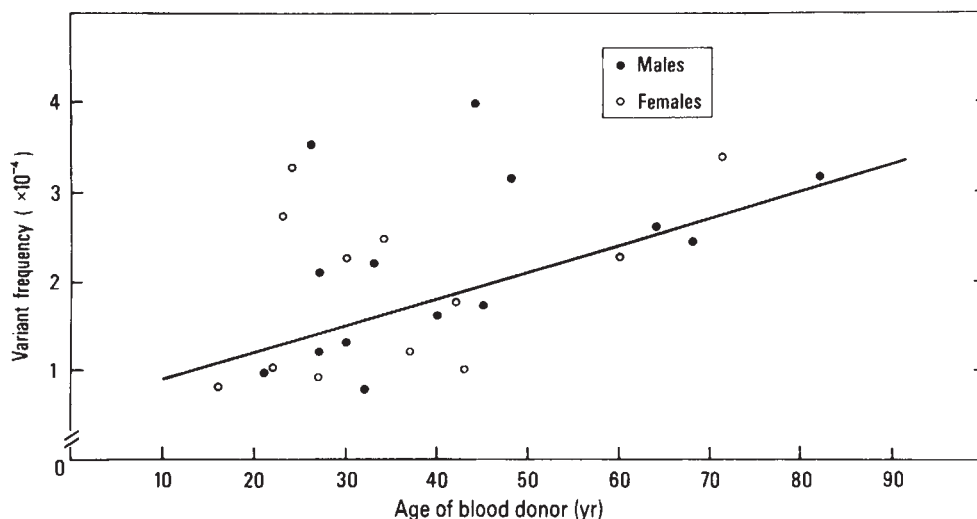
**Fig. 2** AG^r frequencies in blood lymphocytes of individuals of different ages.

Table 2 Variant frequency in human peripheral blood lymphocytes exposed to X rays

Radiation dose	+Azaguanine			-Azaguanine			Variant frequency ($\times 10^{-4}$)
	Labelled cells	Cells counted	Labelling index ($\times 10^{-3}$)	Labelled cells	Cells counted	Labelling index	
Expt I							
Control	72	454,777	0.158319	1,003	5,000	0.2006	7.89 $\pm$ 0.96
50 rad	109	676,089	0.161221	890	5,000	0.1780	9.06 $\pm$ 0.92
100 rad	58	328,998	0.176292	464	5,000	0.0928	19.00 $\pm$ 2.56
200 rad	44	241,749	0.182006	230	5,000	0.0460	39.56 $\pm$ 6.77
400 rad	35	231,676	0.151073	130	5,000	0.0260	58.10 $\pm$ 12.74
Expt II							
Control	89	533,180	0.166923	1,160	5,000	0.2320	7.19 $\pm$ 0.78
40 rad	115	715,109	0.160815	1,020	5,000	0.2040	7.88 $\pm$ 0.77
80 rad	151	849,436	0.177765	820	5,000	0.1640	10.84 $\pm$ 0.96
120 rad	151	726,107	0.207958	610	5,000	0.1220	17.05 $\pm$ 1.56
160 rad	131	556,885	0.235237	480	5,000	0.0960	24.50 $\pm$ 2.37
200 rad	114	510,800	0.223179	320	5,000	0.0640	34.87 $\pm$ 3.88

Two separate experiments were carried out using separated lymphocytes from a single blood donor. The cells were exposed to a graded series of X-ray doses (at 183 rad min^{-1} from a Siemens Stabilipan 300 kV; HVL 3.1 mm Cu at 12 mA with Thoraeus I filter) at the time of culture initiation, were transferred to fresh medium containing PHA, with or without 8-AG, for 40 h at 37 °C and processed as described in Fig. 1 legend.

Induction of AG^r variants following X-ray irradiation

Two groups^{15,17} have studied the induction of TG^r and AG^r mutants following exposure of human fibroblast cultures to X rays and report similar linear dose-response curves and mutation rates of 2.1×10^{-7} and 3.1×10^{-7} per viable cell per rad. At 50 rad the mutation frequency was about twice the spontaneous level and at 200 rad was increased four- to sixfold. In an experiment on AG^r incidence in X ray-irradiated lymphocytes (Table 2), we observed no significant increase following exposure to 50 rad, but did so at higher dose levels. The best fit to a linear dose response gave an induced variant rate of 1.25×10^{-5} per rad, but the data give a better fit to the power law function $y = \alpha + \beta d^n$, with $n = 1.4$. This curvilinearity was investigated further in a second experiment (Table 2) where the inclusion of additional dose points emphasized the curvilinear nature of the response, the data fitting a quadratic with negligible linear term, or a power law function with $n = 2.1$. Assuming $n = 2$, then $\alpha = 6.90 \pm 0.11 (\times 10^{-4})$ and $\beta = 6.81 \pm 0.20 (\times 10^{-4})$.

The X-ray results show that: (1) significant increases in AG^r variant lymphocytes occur following exposure to quite low doses

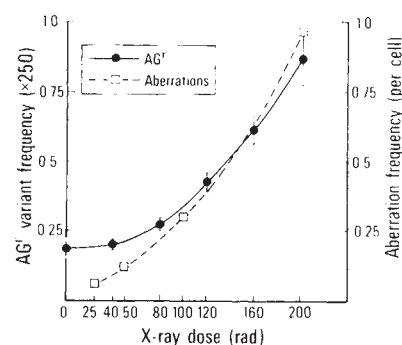


Fig. 4 AG^r variant frequencies per lymphocyte versus X-ray dose (—●—) compared with aberration frequencies in X ray-irradiated lymphocytes (---□---). The aberration frequencies are from Lloyd *et al.*³⁶ and include totals of fragments, centric rings and dicentric, with the dicentric incidence doubled to account for equal numbers of undetected translocations (see ref. 37). The AG^r frequencies have been multiplied by a factor of 250 for direct comparison with the aberration frequencies.

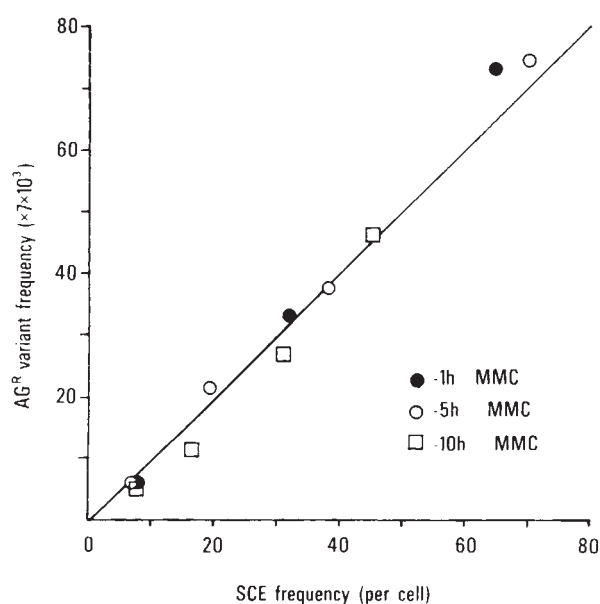


Fig. 3 SCE frequency per cell plotted against AG^r frequency per cell multiplied by 7×10^3 . The close fit of the experimental points to a line of unit slope indicates that over the range of doses and treatment times with MMC there are 7,000 SCE events for every single event resulting in an AG^r phenotype.

of X rays; (2) the dose response is curvilinear over 0–200 rad and approximates to a square law; (3) the kinetics of dose response for the induction by X rays of AG^r variant lymphocytes is remarkably similar to that for the induction of chromosome aberrations in lymphocytes (Fig. 4); (4) the rate of induction of AG^r lymphocytes is some 40 times that for AG^r fibroblasts, but is similar to mutation rates in human/hamster hybrid fibroblasts where mutant fitness does not influence mutant yield³⁴; (5) X ray-induced AG^r variants, in contrast to MMC-induced variants, are unlikely to be due to transcriptional blocks, but are more probably true mutations.

Cox and Masson¹⁵ have shown that some 40% of X ray-induced TG^r fibroblasts have stable X-chromosome aberrations (for example, deletions, translocations) whose location is consistent with the mapped position of the HPRT locus on the X chromosome. It seems probable therefore that most X ray-induced purine analogue-resistant mutants are associated with two types of gross structural change: those resulting in breakage of the X chromosome proximal to the HPRT locus, leading to loss of the gene at mitosis, and those resulting in breakage or damage directly at the locus itself. As the lymphocyte experiments involve no mitosis between X-ray irradiation and AG^r expression, direct damage to the HPRT locus seems the more likely. The relationship between the induction of chromosome aberrations in lymphocytes and X-ray dose is curvilinear^{35,36}, with exchange aberrations increasing as approximately [dose]² and deletions as approximately [dose]^{1.4}. Much of the published data relate only to unstable aberrations, but in Fig. 4 published dose-response data have been corrected to include all symmetrical interchange events, and the response curves for AG^r

and aberration induction are seen to be similar in shape, with roughly one AG^r event for every 250 or so aberrations. Breakage sites are distributed in proportion to chromosome length so that some 2.5% occur in the X chromosome; thus, there are approximately six visible X-chromosome breaks for every variant induced. Our own studies of the total aberration spectrum in G- and R-banded lymphocyte chromosomes³⁷ from cells exposed to 200 rad of X rays reveal 150 breakage points in every 100 cells. At this dose there are 35 AG^r variants in 10⁴ cells, or 35 variants per 15,000 breaks, that is, ~10 visible X-chromosome breaks for each variant.

Concluding comments

We have shown that the incidence of peripheral blood lymphocytes resistant to normally toxic levels of 8-azaguanine (AG^r cells) increases with the age of the blood donor, is independent of sex and may well reflect the presence of cells with deletions of the terminal region of the X chromosome which contains the HPRT locus. *In vitro* exposure to MMC markedly enhances the incidence of AG^r lymphocytes in a dose-dependent fashion and the rate of increase parallels the increasing incidence of induced SCEs. The high frequency of these MMC-induced AG^r cells and the fact that purine analogue resistance is expressed before the

cells pass through a DNA replication phase, argue against a mutational origin for these cells. Their close correlation with the induction of SCEs is, however, considered to indicate that both AG^r and SCE induction by MMC are a consequence of interaction of the mutagen with DNA and it is suggested that the AG^r variants simply represent cells in which the large MMC-guanine adducts have prevented normal transcription of the HPRT locus to give functional HPRT.

X-ray damage, which does not require misreplication at the S phase to result in mutational change, is also effective in yielding AG^r cells. The dose response *in vitro* is curvilinear and is very similar to that for X-ray-induced chromosome aberrations in lymphocytes. As chromosome, or fragment, loss cannot intervene between the time of irradiation and the expression of the AG^r phenotype, it is argued that X-ray-induced AG^r cells may be true mutants and our evidence indicates that for every induced AG^r event in a cell population there are about 10 visible induced breaks in an active X chromosome.

Finally, we note that the methods used to detect and count purine analogue-resistant variants in human lymphocyte populations are amenable to automation by flow cytometry, so that this approach may offer a powerful method for studying the effects of mutagens on human cells.

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Immunocytochemical localization of glutamic acid decarboxylase in monkey striate cortex

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Neuronal cell bodies and synaptic terminals positive for glutamic acid decarboxylase, the enzyme responsible for synthesizing γ -amino butyric acid, have been located by immunocytochemical staining in all layers of the macaque monkey cortex. In layers II and III the staining pattern of periodic dots is identical with that seen in sections stained for cytochrome oxidase. The rows of dots run parallel with the ocular dominance columns, suggesting that the labelled neurones are preferentially related to each eye.

γ -AMINOBUTYRIC ACID (GABA) functions as an inhibitory neurotransmitter in the mammalian cerebral cortex including the visual cortex^{1–4}. We have localized the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) in monkey striate cortex and mapped the distribution of neuronal cell bodies and synaptic terminals positive for GAD (GAD⁺). Primate striate

cortex has the advantage of being precisely laminated and has well characterized inputs and outputs⁵. Standard light microscopic immunocytochemical techniques were used⁶, with an antiserum to mammalian GAD which has been extensively characterized^{7–9}. We have found that all cortical layers contain both neurones and synaptic terminals which are GAD⁺, but in

different quantities. No variation in the distribution or number of GAD⁺ terminals was found in layer IVC which would correspond to the ocular dominance columns¹⁰, but in layers II and III a 375- μ m periodic repeat of GAD⁺ dots arranged in rows was found. When the same brains were histochemically stained for the mitochondrial enzyme cytochrome oxidase^{11,12}, the laminar density and distribution of cytochrome oxidase staining was the same as for GAD and an identical pattern of dots in rows was found in layers II and III.

Experimental procedure

Primary visual or striate cortex from four normal *Macaca fascicularis* monkeys was studied. In one animal the left eye was injected with 3 mCi of a mixture of ³H-proline and fucose 14 days before the animal was killed, to label the cortical ocular dominance columns by transynaptic transport¹⁰. In two animals the striate cortex received 1- μ l injections of colchicine (10 μ g μ l⁻¹) 1 day before they were killed to increase the GAD content of the neuronal cell bodies¹. GAD immunocytochemical and cytochrome oxidase staining methods are described in the legends of Figs 1 and 2.

In sections cut perpendicular to the cortical surface (Fig. 1b), the layers were strikingly outlined in the anti-GAD-incubated sections; sections incubated without specific anti-GAD serum showed no staining (Fig. 1a). All layers contained GAD staining

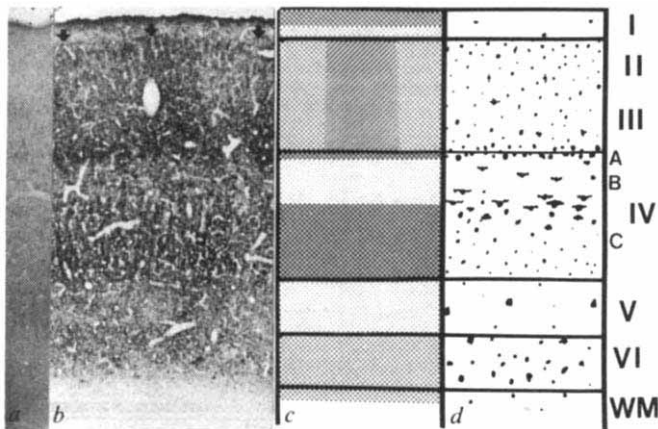


Fig. 1 *Macaca* monkey striate cortex with the layers numbered according to Lund¹³ on the far right of the figure. All monkeys were deeply anaesthetized and then perfused intracardially with phosphate-buffered 4% paraformaldehyde, pH 7.4, or a phosphate-buffered mixture of 4% paraformaldehyde, lysine and sodium periodate, pH 7.4. The brain was postfixed for 4–24 h, small pieces of cortex were sunk in 30% sucrose in phosphate buffer and serial frozen sections cut both perpendicular and parallel to the cortical surface. For immunocytochemistry, sections were incubated at 25 °C overnight in anti-GAD serum^{7,8} diluted 1/150 in phosphate-buffered saline, pH 7.4 containing 0.3% Triton and 1% normal sheep serum. This saline mixture was used subsequently for all antisera dilutions. After thorough rinsing, the sections were incubated in sheep anti-rabbit IgG serum diluted 1/10 for 30 min at 37 °C, and then in rabbit peroxidase–antiperoxidase⁶ diluted 1/50 for 30 min at 37 °C. The peroxidase was visualized by reacting the sections for 20 min at 25 °C in diaminobenzidine and hydrogen peroxide. Some sections were then lightly counterstained with thionine. Control sections were identically processed with the specific anti-GAD serum replaced by normal rabbit serum. *a, b*, Frozen sections processed for immunocytochemistry where the primary serum incubation used was (*a*) normal rabbit serum or (*b*) specific anti-GAD serum. In *b* all layers contain GAD⁺ synaptic terminals, but the amount varies between layers. In layers II and III periodic 375- μ m variations in density (*b*, arrows) are found. $\times 39$. *c*, Schematic representation of the differences between layers in the density of GAD⁺ synaptic terminals. *d*, Schematic representation of the laminar distribution of GAD⁺ neuronal cell bodies; this drawing is based on many different sections from colchicine-pretreated cortex.

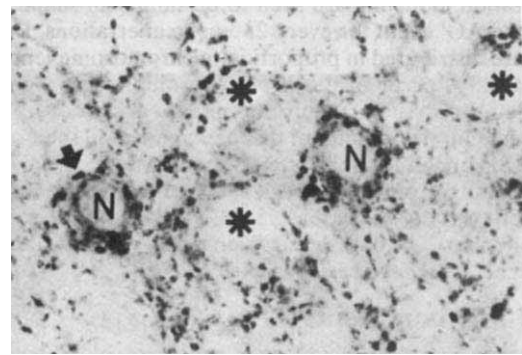


Fig. 2 Photomicrograph from layer IVC in a section reacted with specific anti-GAD serum. Many cell bodies do not stain (*) but are outlined by GAD⁺ terminals. Two GAD⁺ cell bodies show stained cytoplasm but an unstained nucleus (N) and are surrounded by GAD⁺ terminals (arrows). The neuropile also contains many GAD⁺ beaded processes and terminals. $\times 840$.

but the intensity of labelling varied with the layer. The shifts in density corresponded to the laminar boundaries^{5,13} identified in thionine-stained sections. Under higher power (Fig. 2), the GAD staining was localized to cell bodies, beaded processes and small round densities which resembled synaptic boutons. Ribak¹ has shown in electron micrographs of GAD-stained rat visual cortex that these densities are synaptic terminals synapsing on cell bodies and dendrites. Because our preliminary electron microscopy has confirmed this finding for the monkey, we shall provisionally call these small densities terminals. There was no difference in laminar staining of central to mid-peripheral retinal representation in cortex; far-peripheral cortex was not examined.

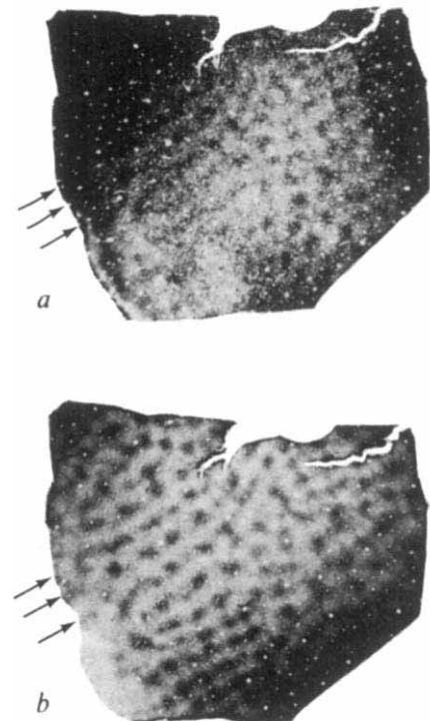


Fig. 3 *a*, A section cut parallel to the cortical surface passing through layers II and III which has been reacted in specific anti-GAD serum. An orderly pattern of dots aligned into rows can be seen (arrows). $\times 8.5$. *b*, An adjacent section stained for cytochrome oxidase by a 16-h incubation at 37 °C in phosphate-buffered diaminobenzidine and cytochrome *c*^{11,12}, pH 7.4. The same pattern of dots aligned in rows is found. Compare the rows marked by the arrows in the two sections; >80% of the dots in these two sections directly superimpose on one another. $\times 8.5$.

Distribution of GAD⁺ terminals

As judged by both eye and photodensitometry, the upper halves of layers I, IVA and IVC were the most heavily labelled, II, III and VI slightly less so, and lower I, IVB and V were quite lightly labelled (Fig. 1*b,c*). The labelling in layer I was uniformly distributed and consisted of small, round GAD⁺ terminals in no obvious pattern. In all the other layers the labelling showed two patterns: GAD⁺ beaded processes run parallel or perpendicular to the cortical surface, and GAD⁺ terminals encircle both unstained and GAD⁺ cell bodies (Fig. 2). Most of the GAD⁺ terminals are ~1 µm in size, but occasionally there are clusters of much larger, 3–5-µm terminals. GAD⁺ terminals continue at least 50 µm into the white matter below the most inferior layer VI neurones.

Some GAD-reacted sections from the ³H-proline and fucose eye-injected monkey were coated for autoradiography, using Kodak NTB2 emulsion and 3–4-month exposures. Ocular dominance columns were apparent in layer IVC, but no

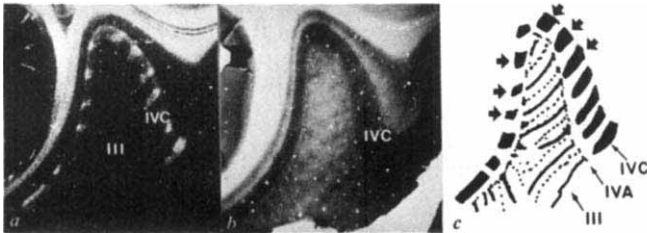


Fig. 4 Sections of striate cortex cut parallel to the cortex surface from the monkey whose left eye had been injected intravitally with 3 mCi of equal parts ³H-proline and fucose 14 days before it was killed. The sections were first stained for cytochrome oxidase and then processed for autoradiography. In *a* the labelled ocular dominance columns from the left eye are visible in the dark-field photomicrograph as periodic repeats in IVC. In *b* this bright-field picture shows the same cytochrome oxidase-stained section. Layer IVC is darkly stained. Layer III and above contains periodic rows of dots. *c*, A drawing based on a short series of these doubly processed sections. Layer IVC contains alternating left eye-labelled (solid black), right eye-unlabelled (white) ocular dominance columns. Beginning at layer IVA rows of cytochrome oxidase (or GAD⁺) dots lie at regular 350–400-µm intervals. The rows are schematically represented here; rows related to labelled columns are solid lines while rows related to unlabelled columns are dotted lines. There is one row for each column, running parallel to the axis of the column approximately along its centre. The three ocular dominance columns marked by arrows each became a continuous band in this series; all three ran parallel to the rows of dots. The ocular dominance columns and rows of dots related to the unlabelled eye were stained slightly darker in this monkey, possibly indicating some complication in the eye injection which was reflected by decreased cytochrome oxidase activity in cortex.

differential density of GAD⁺ terminals could be seen at either the column edges or centres, confirming the results from the other three animals. In all four monkeys a periodic repeat of GAD⁺ density was seen in layers II and III under low power (Fig. 3*a*; also see Fig. 1*b* arrows). This pattern consisted of fairly discrete dots arranged in rows formed by less dense connections between the dots. Along the rows the dots had a centre-to-centre spacing of 466 ± 75 µm with the rows 378 ± 55 µm apart. Under higher power it was apparent that the dots were formed by GAD⁺ cell bodies and a high density of GAD⁺ terminals. In adjacent sections stained for cytochrome oxidase (Fig. 3*b*), an

identical pattern of dots in rows was found. More than 80% of the GAD⁺ dots superimposed on the cytochrome oxidase dots in an adjacent section. When cortex sections from the ³H-proline/fucose eye-injected monkey were first stained for GAD or cytochrome oxidase and then processed for trans-synaptic autoradiography, the rows of dots in layer III ran parallel to the centres of the ocular dominance columns in layer IVC (Fig. 4).

Distribution of GAD⁺ cell bodies

Neuronal cell bodies have been considered to be specifically stained if they show an unstained nucleus with neuronal characteristics and a heavily stained cytoplasm with a sharp cellular outline (Fig. 2*N*). In the two brains which received no colchicine, there were very few GAD⁺ cell bodies in layers I–IVC, but in layers V and especially VI, regularly spaced GAD⁺ neurones 15–20 µm in diameter were found. Small GAD⁺ cell bodies 8–10 µm in diameter lay in the lower edge of VI or in the white matter just under VI. In the two brains receiving colchicine injections, there was a striking increase in the number of GAD⁺ cell bodies, especially in layers I–IV. Even if our material is an underestimate because of the technical limitations inherent in these *in vivo* colchicine injections, our experiments indicate that monkey striate cortex contains many GAD⁺ neurones ranging in size from 9 to 30 µm. GAD⁺ neurones occur in every cortical layer and most appear to be various types of stellate neurone. Their distribution is schematically presented in Fig. 1*d*. In all layers some GAD⁺ neurones receive GAD⁺ terminals onto their cell bodies or dendrites. Most unstained cell bodies are outlined by GAD⁺ terminals in all layers.

Conclusions

Three findings emerge from this study. (1) The striate cortex of *Macaca* monkeys is very rich in both GAD⁺ cell bodies and synaptic terminals. Unlike rat visual cortex¹, there are clear laminar differences in the distribution of both GAD⁺ cell bodies and synapses. (2) There is a striking similarity between GAD distribution and cytochrome oxidase staining. Wong-Riley^{11,12} first pointed out the good correlation between cytochrome oxidase staining, GABA or GAD content and iron concentration in regions like cerebellum and basal ganglia. She suggested that inhibitory GABAergic cells are mitochondria-rich, energy-dependent cells which stain especially heavily for the haem enzyme cytochrome oxidase. This agrees with an earlier study¹⁴ where the nerve terminals which became labelled after exposure of rat brain homogenates to ³H-GABA were characterized by a high density of mitochondria within their cytoplasm. Our data support these suggestions and provide more direct evidence that cytochrome oxidase staining may be largely localized to GABAergic neurones. (3) The cytochrome oxidase and GAD⁺ pattern of rows of dots in layers II and III is identical to that shown in *Macaca* striate cortex using 2-deoxyglucose labelling¹⁵ in which similar rows of dots are parallel to the ocular dominance columns. In this study, sections doubly processed for GAD or cytochrome oxidase and trans-synaptic autoradiography also reveal that each row of dots is aligned parallel to the centre of each ocular dominance column. It therefore appears that there are rows of GAD⁺ inhibitory neurones in supragranular *Macaca* striate cortex which may be preferentially related to each eye.

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LETTERS TO NATURE

Measurements of a solar flare-generated shock wave at $13.1 R_0$

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Most information on interplanetary shock waves generated by solar flares has come from *in situ* measurements near 1 AU and beyond¹⁻³, and little is known about the structure near the Sun. We report here the first measurements of the structure of wind speed, electron density and electron density fluctuations of a shock wave propagating through the acceleration region of the solar wind. The radio-scattering observations consisting of spectral broadening, mean phase and amplitude scintillations were made on 18 August 1979, $13.1 R_0$ east of the Sun and near the ecliptic plane using the Voyager 1 2.3- and 8.4-GHz radio signals. The results show that the shock wave had a shock speed of $\sim 3,500 \text{ km s}^{-1}$. When compared with the average speed based on its transit time to 1 AU, it is seen that substantial deceleration took place as the shock wave propagated outwards from the Sun, a result that is consistent with a blast wave^{1,4}.

Figure 1 shows the view of Voyager 1, which was 7.03 AU from the Earth, and the Sun as seen from the Earth on 18 August 1979. It is clear that the Voyager 1 radio path was ideally positioned for studying the shock wave which was apparently generated by a solar flare located at N09 E90 (ref. 5). The longitudinal extent of the interplanetary disturbance was large because the shock was measured 90° away near 1 AU by the ISEE-3 magnetometer at 05.50 UT on 20 August (E. J. Smith, personal communication). The average shock speed based on the transit time to ISEE-3 is $1,053 \text{ km s}^{-1}$. A type II radio burst associated with this shock wave was also tracked in the 30 kHz–2 MHz frequency range by the radioastronomy experiment on ISEE-3 (ref. 5).

The results presented here are derived from the bandwidth-reduced digitally recorded data of the 'open-loop' receivers at the 64-m NASA Deep Space station located in Goldstone, California⁶. The 2.3- and 8.4-GHz signals were recorded in bandwidths of 1 and 3 kHz, respectively. For studying the amplitude scintillations and mean phase, these signals were further reduced to 100 Hz using a digital phase lock loop procedure⁷.

The radio measurements and the inferred results are summarized in Figs 2 and 3, respectively. Shock arrival occurs at $\sim 15.01 \text{ UT}$, and based on the transit time from the Sun to $13.1 R_0$, we obtain a shock speed of $3,509 \text{ km s}^{-1}$. The time histories of spectral broadening^{8,9} of bandwidth B at 2.3 and 8.4 GHz for 1-min integration times are shown in Fig. 2a. B is the full width between frequencies enclosing half the area under the radio-frequency spectrum. The pre-shock bandwidth at 2.3 GHz is $\sim 1 \text{ Hz}$ and is consistent with previous measurements of the quiet solar wind⁸. During shock passage the bandwidths increase

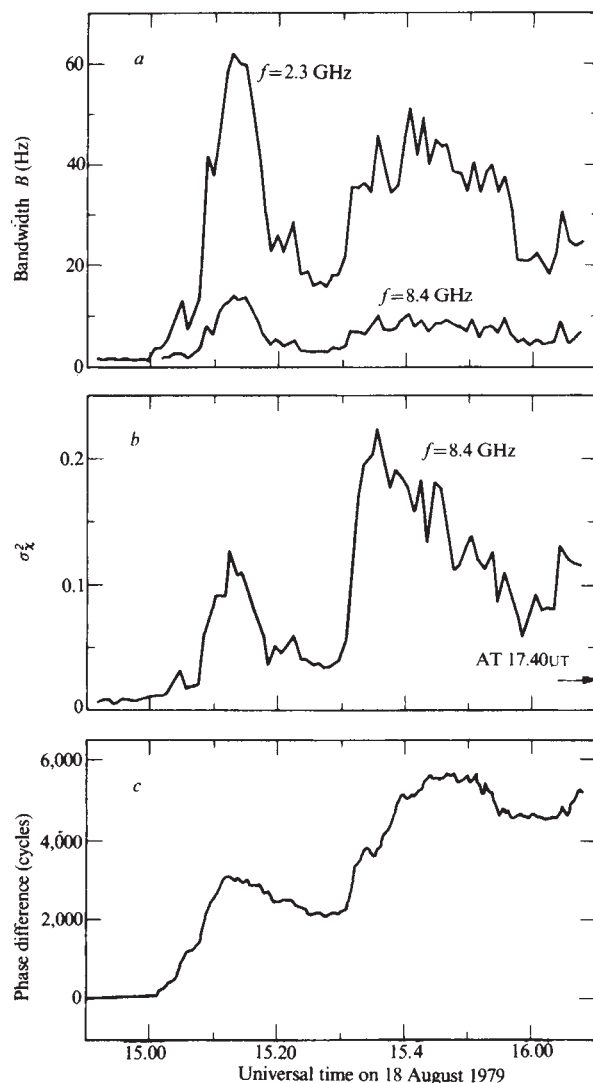


Fig. 2 Voyager 1 radio-scattering observations as a function of UT on 18 August 1979. *a*, Spectral broadening bandwidth B at 2.3 and 8.4 GHz. *b*, Variance σ_x^2 of the log-amplitude scintillations at 8.4 GHz. The arrow shows the σ_x^2 level at 17.40 UT. *c*, Difference between the 2.3 GHz and $3/11$ of the 8.4-GHz phases.

considerably and two regions of enhanced bandwidth are observed. Although such transient events are rare during the solar minimum portion of the solar cycle⁸, they have been observed previously around the solar maximum^{9,10}. By examining the frequency dependence⁸ of B at 2.3 and 8.4 GHz we find that the average spectral index p of the three-dimensional power-law spatial wavenumber spectrum is 3.66 with a standard deviation of 0.11. The density spectrum, which is essentially Kolmogorov, is steeper than the quiet Sun spectrum for similar scales in this region of the solar wind¹¹, but consistent with other measurements during disturbed solar wind conditions¹⁰. Bandwidth increases are caused by enhancements in turbulence (electron density fluctuations) or acceleration of the solar wind⁸. These two effects can be separated by analysing the amplitude scintillations.

The time history of the variance, σ_x^2 , of the log-amplitude scintillations at 8.4 GHz is shown in Fig. 2b. For weak scintillations ($\sigma_x^2 \ll 1$), σ_x^2 is related to the scintillation index m by $\sigma_x^2 = m^2/4$. The amplitude scintillations at 2.3 GHz are not

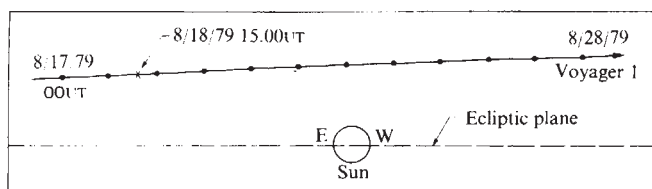


Fig. 1 View of Voyager 1 and the Sun as seen from the Earth on 18 August 1979.

useful during shock passage because they are strong and saturated. The structure constant c_n of the refractive index fluctuations which characterizes the strength of the turbulence has been inferred from the 8.4-GHz scintillations and is shown in Fig. 3a. The corresponding scale for c_{n_e} , the structure constant for electron density fluctuations, is shown on the right. A spherical wave radio analysis has been used to interpret the scintillations¹². Before shock arrival c_n is assumed to vary as R^{-2} (R is heliocentric distance) along the radio path¹³. As the shock crosses the radio path, we assume that the contribution to σ_x^2 along the radio path outside the shock remains the same, and attribute the excess of the observed σ_x^2 to a uniform distribution of c_n within the shock. The extent of the radio path traversed by the shock is defined by assuming that the shock is spherical in shape, emanates from the surface of the Sun and travels at a speed of $3,509 \text{ km s}^{-1}$. Figure 3a indicates that there are two regions of enhanced small-scale electron density fluctuations, corresponding to the two regions of increased spectral broadening. In these regions the turbulence representing scale sizes smaller than the Fresnel size (73 km at 8.4 GHz) increases by a factor of 3.7.

Two methods are used to deduce the solar wind flow speed (v) profiles shown in Fig. 3b. The log-amplitude scintillations are

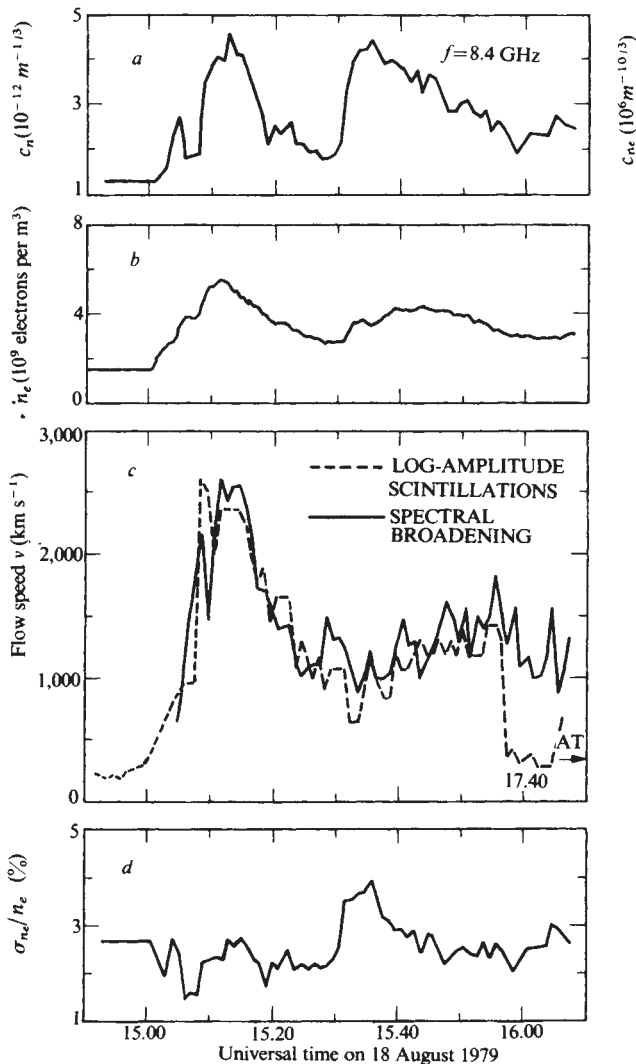


Fig. 3 Measurements of the shock wave inferred from the radio observations of 18 August 1979 shown in Fig. 2. *a*, Strength of turbulence as characterized by the structure constants c_n and c_{n_e} of refractive index and electron density fluctuations, respectively. The results for c_n are for 8.4 GHz. *b*, Electron density n_e . *c*, Solar wind flow speed, v , as deduced using two methods (see text). The arrow corresponds to the flow speed measured at 17.40 UT. *d*, Ratio σ_{n_e}/n_e in per cent, where σ_{n_e} is the r.m.s. electron density fluctuation in scales smaller than the X-band Fresnel scale (73 km).

spectrum analysed in 1-min intervals so that velocity can be determined for these times from the location of the 'knee' or Fresnel cutoff using a spherical wave radio analysis¹⁴. Although this method has considerable uncertainties when applied to quiet observations near the Sun because of the rounding of the knee due to fluctuations of the order of the mean¹¹, it is useful in the case of the shock wave because the mean speed is large and velocity fluctuations are not significant. Generally, the correlation of multiple station intensity scintillations is the preferred radio method for measuring solar wind speed^{11,15}. Near the shock front, two knees are present in the spectra, one due to the shock and the other to the slower ambient wind outside the shock. In these cases the higher velocity is always plotted. In a few instances around 15.00 UT, velocities could not be estimated due to the smearing of the knee by a rapidly rising velocity. At 15.58 UT the wind speed decreases quite abruptly. In this region the spectra again show two peaks. For these cases, however, the slower speed is chosen because it is clear from the level of the spectrum that it is due to a slowing of the flow speed along a radial cut through the shock and not to the slower ambient solar wind outside the shock.

The second method for estimating velocity involves using the measurement of σ_x^2 to remove the effect of turbulence from the spectral broadening observation in order to solve for velocity. This method requires that p be known. As this has been determined to be Kolmogorov, we can use the relationships that have been derived for a spherical wave¹². The velocity profile inferred using this method is also shown in Fig. 3c. The agreement between the two independent methods is remarkable. When two velocities are present, the spectral broadening technique senses the faster one. This explains why this method gives correct estimates near the shock front but incorrect estimates beyond 15.56 UT.

As the shock crosses the Voyager radio path the flow speed rises sharply from 200 to $2,600 \text{ km s}^{-1}$, a factor of 13. Near the peak the velocities are fairly well determined and we estimate that the $2,600 \text{ km s}^{-1}$ measurement is accurate to within 20%. Elsewhere, the differences between the two curves in Fig. 3b probably represent the uncertainties in the velocity determination. Because the gross variation in velocity is substantial, it is easily recognizable and measured in spite of the uncertainties.

The final observation is phase at 2.3 GHz shown in Fig. 2c. This is formed by taking the difference between the S band and 3/11 (the frequency ratio of S and X bands) of the X-band phases¹⁶. During the phase lock loop procedures mentioned earlier, the S-band phase is non-coherently estimated using the X-band phase as a guide in the loop. In this way, any 2π ambiguities due to tracking errors are minimized. Because 'absolute' phase was not measured, Fig. 2c represents only the change in total electron content. We have deduced the electron density profile in Fig. 3b in the following manner. At $13.1 R_0$, we assume that the electron density $n_e = 1.5 \times 10^9$ electrons per m^3 . Before shock arrival we calculate the total electron content assuming a R^{-2} dependence for n_e . As the shock crosses the path we assume that the contribution to the total electron content outside the shock remains the same and attribute the excess of the observed electron content to a uniform distribution of n_e inside the shock. The extent of the shock is defined in the same way as that used for deducing c_n . Note that this inversion scheme used to obtain both c_n and n_e is most accurate near the shock front. Further back in the wake it becomes less accurate because, strictly speaking, c_n and n_e are not uniformly distributed along the radio path inside the shock. Nevertheless, it is a reasonable approximation and the general profiles for c_n and n_e should not be significantly affected. We note that the peak density increases by a factor of ~ 3.7 over the pre-shock value and is consistent with the theory for a strong shock¹⁴. As expected for a shock wave, the peak in the turbulence characterized by c_n lags the peak in mean electron density.

Using the Rankine-Hugoniot relationships¹⁴, pre-shock values of $n_e = 1.5 \times 10^9$ electrons per m^3 and $v = 200 \text{ km s}^{-1}$, post-shock values of $n_e = 5.5 \times 10^9$ electrons per m^3 and

$v = 2,600 \text{ km s}^{-1}$, we compute a shock speed of $3,500 \text{ km s}^{-1}$, which is consistent with the shock speed of $3,509 \text{ km s}^{-1}$ obtained from the transit time to $13.1 R_0$. According to time delay radio measurements¹⁷, electron densities in the solar wind at $13.1 R_0$ may be as high as 3×10^9 electrons per m^3 . We have derived density profiles similar to Fig. 3b corresponding to 2×10^9 and 3×10^9 electrons per m^3 , and obtained shock velocities of $3,660$ and $4,320 \text{ km s}^{-1}$, respectively. The fact that these shock speeds are higher than $3,500 \text{ km s}^{-1}$ indicates that some acceleration could have taken place between the Sun and $13.1 R_0$. This behaviour has been observed in white light coronagraph measurements¹⁸, although shock speeds as high as $3,500 \text{ km s}^{-1}$ seem to be very rare^{18,19}. Questions do arise about the location of the shock fronts in the white light coronagraph pictures²⁰ and simultaneous measurements with radio-scattering observations would be desirable. It is clear that the shock speed inferred from the Rankine-Hugoniot relationships has a rather high uncertainty because of two factors. First, the 20% accuracy in the flow-speed estimate translates approximately into a 20% accuracy in the deduced shock speed. Second, the uncertainty in the pre-shock electron density also leads to inaccuracy in the inferred shock speed. For example, by increasing the pre-shock electron density to 2×10^9 and 3×10^9 electrons per m^3 we find that the shock speed increases by 5 and 23%, respectively.

We have plotted σ_{ne}/n_e (σ_{ne} is r.m.s. electron density fluctuations) in per cent in Fig. 3d. In this case σ_{ne} has been computed for scale sizes smaller than the Fresnel size at 8.4 GHz (73 km). The fluctuations amount to several per cent before and after shock passage. It is interesting that this ratio shows a noticeable increase at about 15.35 UT. This represents the start of the second region of enhanced amplitude scintillations, and may correspond to the turbulent 'driver gas' of the shock wave. In contrast, the first region of enhanced amplitude scintillations corresponds to the compressed region of the shock wave.

The radio data end at about 16.08 UT before the completion of shock passage. However, some data were collected for a few minutes around 17.40 UT and yield a wind speed of 320 km s^{-1} and $\sigma_x^2 = 0.0225$ at 8.4 GHz . These values, indicated by arrows in Figs 2b and 3c, show that the solar wind has almost returned to its pre-shock state.

It is clear that radio-scattering measurements using spacecraft radio signals complement similar measurements using natural radio sources²¹ and constitute a powerful tool for identifying and studying the detailed structure of coronal transients and disturbances. Plasma measurements of the 18 August shock wave were made by ISEE-3 at 1 AU. Information on shock speed beyond $13.1 R_0$ but still close to the Sun will also be available from the ISEE-3 type II radio-burst observations. Future studies will correlate these measurements with the present results and will undoubtedly lead to an improved understanding of the evolution and deceleration of shock waves in the solar wind.

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Chemical composition of the atmosphere of Venus

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Measurements onboard the Venera 11, 12 (refs 1-4) and Pioneer Venus^{5,6} spacecrafts stimulated us to study the chemical composition of the subcloud atmosphere of Venus in terms of the thermochemical equilibrium calculations, comparison of typical mixing and chemical times and a rule of height-independent element mixing ratio in the absence of condensation⁷. The photochemistry of the atmosphere down to 50 km was calculated using transport effects and number densities of CO_2 , H_2O , HCl , SO_2 and CO at the lower boundary and rate coefficients of 102 reactions. These reactions include catalytic cycles of COCl and COCl_2 which accelerate O_2 destruction and CO_2 formation. Altitude profiles of 27 components agree well with those measured in the upper and middle atmosphere. H_2O and SO_2 mixing ratios are very similar and sharply decrease at 60 km due to SO_2 photolysis and sulphuric acid formation. Calculations show that sulphuric acid and sulphates are the main components of the second and third modes of particle size distribution in the upper and middle cloud layers. The lower cloud layer may consist of AlCl_3 and FeCl_3 .

A model of atmospheric composition should produce vertical profiles of all atmospheric components from data on the abundance of parent molecules. This requires a knowledge of the chemical rate constants k_i , the values of temperature T and the eddy mixing coefficient K . As those constants are known in a few cases only, the number of components considered has to be limited and aeronomic analyses restricted to those parts of the atmosphere where these limitations apply.

The subcloud atmosphere (0-50 km) has a fairly high temperature and pressure (740 K and 90 atm near the surface) and is not exposed to solar UV radiation. Although thermochemical processes involving stable molecules dominate the region their rate constants are generally unknown. Hence we calculated only the thermochemical equilibria from the free enthalpy of the components. The comparison of the mixing times $\tau_m = H^2/K$ where H is the scale height, and of the chemical equilibrium, $\tau_c = (\sum_i k_i n_i)^{-1}$ where n_i is the concentration of a reagent considered, shows that mixing dominates chemical reactions in the whole lower atmosphere. The catalytic effect of the ground tends to accelerate chemical processes. For the order of magnitude comparison we used $K \approx 10^4$ - $10^6 \text{ cm}^2 \text{ s}^{-1}$, $k_i \approx 10^{-10} \exp(-25,000/T) \text{ cm}^3 \text{ s}^{-1}$, mixing ratio $f_i \approx 10^{-5}$; chemical processes on the ground were assumed to be accelerated by two orders of magnitude.

The atmospheric composition should then be approximately constant throughout the subcloud and can be determined by the thermochemical equilibrium near the surface (Qyama *et al.*⁵ assumed thermochemical equilibrium throughout the subcloud atmosphere). Calculations were based on the first data from Venera 11, 12 and Pioneer Venus experiments. The initial data chosen were concentrations of CO_2 , N_2 , SO_2 for which Soviet and US measurements had given similar results, concentrations of H_2O and S_2 (our estimate based on the spectroscopic

Table 1 The calculated composition of the subcloud atmosphere of Venus

Gas	CO ₂	N ₂	SO ₂	H ₂ O	S ₂	HCl	HF	CO	COS	H ₂ S	H ₂
Mixing ratio f_i	0.96	3.4×10^{-2}	1.3×10^{-4}	2×10^{-4}	1×10^{-7}	1×10^{-6}	1×10^{-8}	1.5×10^{-5}	2×10^{-5}	3×10^{-7}	2×10^{-8}

measurements⁴), of HCl and HF from ground-based observations⁸. Our calculations are summarized in Table 1 (inert gases are not taken into account).

Components whose calculated mixing ratio was lower than 10^{-10} are not given in Table 1.

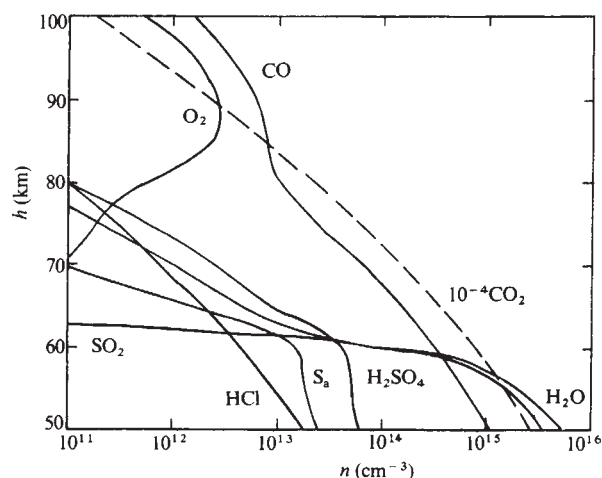
Our calculations and the related estimates are confirmed by further processing of the observational data. The height variation of f_{CO} was determined from Venera 12 data³ for which f_{CO} ranges from 3×10^{-5} above 36 km to 1.5×10^{-5} near the surface. Gas chromatography measurements on Pioneer Venus⁵ first indicated that CO was not present in the lower atmosphere, but then produced a similar dependence on height. Small amounts of H₂S and H₂ initially suggested by Venera 12 data have not been confirmed, while the indication of COS seems reliable³.

Similar calculations based on the data of H₂O and O₂ mixing ratios measured by the gas chromatograph on Pioneer Venus gave an abundance of H₂SO₄ which was much higher than the bulk of the cloud layer.

We now consider the experimentally-obtained height dependences of mixing ratios. Here we take into account that if condensation is absent in a certain atmospheric region and if eddy mixing dominates the molecular diffusion, the relative abundances of elements should be height-independent. This enables the altitude dependences observed to be considered qualitatively.

The height dependence of CO might explain its gradual conversion into COS with decreasing height. The sum $f_{\text{CO}} + f_{\text{COS}} = 3.5 \times 10^{-5}$ agrees well with the spectroscopic measurements⁸ of CO near the top clouds; f_{COS} should decrease with height and be $\sim 10^{-6}$ above 36 km. Its decrease should be accompanied by a slight ($\sim 10\%$) increase of f_{SO_2} up to cloud deck. But in this case f_{COS} should be $5-10 \times 10^{-6}$ at 22 km, which exceeds the upper limit of 2×10^{-6} obtained by the third sample of Pioneer Venus gas chromatography. Because the drastic variation of sulphuric compound abundance at 20-25 km recorded by the Pioneer Venus mass spectrometer⁶ was an experimental artefact it was ignored.

It is difficult to explain height variations of $f_{\text{H}_2\text{O}}$ (2×10^{-4} in the cloud layer and 2×10^{-5} near the surface) deduced from the spectroscopic measurements on Venera spacecraft⁴. While spectroscopic measurements of $f_{\text{H}_2\text{O}}$ seem more convincing than the data of Pioneer Venus gas chromatography⁵ where there could be perturbation effects from the spacecraft (values of $f_{\text{H}_2\text{O}} = 1-5 \times 10^{-3}$ were obtained), we believe that errors in the measurements⁴ and the initial data used for interpretation do

**Fig. 2** Composition of the atmosphere of Venus between 50 and 100 km.

not preclude the possibility of $f_{\text{H}_2\text{O}}$ being independent of height. The other possibility is the presence of some hydrogen-bearing gas with mixing ratio $\sim 10^{-4}$.

We calculated the model composition in the atmosphere down to 50 km by solving a set of differential equations which describe the behaviour of 27 components (CO₂, H₂O, HCl, SO₂ and the products of their photolysis) involved in 102 reactions and include transport effects.

As our model takes into account both chlorine and sulphur compounds, the number of reactions compared with previous calculations⁹⁻¹¹ almost doubled. Our model uses an approximate description of the processes of radiation and matter transport in the cloud layer. We have developed a more universal method of imposing boundary conditions on daughter components, its special cases being photochemical and diffusion equilibrium.

We have considered not only chlorine oxides but also COCl and COCl₂ participating in cycles which are essential to O₂ destruction and CO₂ formation. Reactions of COCl and COCl₂ formation and removal and their rate coefficients¹² are shown in Table 2.

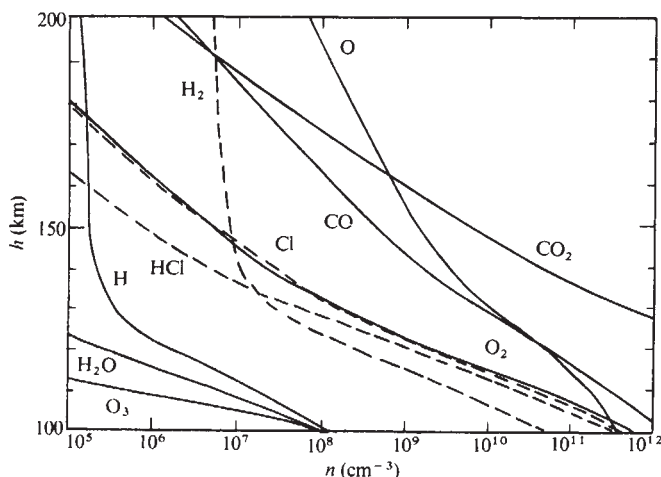
The reaction between H and Cl₂ provides the major sink for odd H* and Cl*. Our model also includes processes of sulphuric acid and sulphur aerosol formation from SO₂, four types of excited components and a long series of reactions.

Figure 1 shows the calculated composition of the upper atmosphere. The results of Pioneer Venus mass-spectrometric^{13,14} and optical¹⁵ measurements agree well with the calculations. Taking into account strong diurnal variability (our calculations were made for solar zenith angle 60°) the comparison should be done for the altitudes at which CO₂ number densities are equal. Figure 2 shows the composition of the atmosphere and the cloud layer at 50-100 km, concentrations of many of minor components with $n < 10^{11} \text{ cm}^{-3}$ are not included there. Figure 3 shows number densities of chlorine compounds. As we have not got room for a detailed analysis of all the components, we briefly describe some typical features of the H₂O, SO₂, H₂SO₄, aerosol sulphur S_a and O₂ distributions for which data are available.

The net photochemical transformation of SO₂ in the presence of H₂O can be described by



for 85% acid drops. Hence, the profiles of SO₂ and H₂O should be similar within most of the cloud layer and their concentrations at 50 km should differ by a factor of 1.3-1.8 for 80-85% acid drops. Our profiles agree well with measurements^{3,4} on

**Fig. 1** Composition of the upper atmosphere of Venus. Dashed lines show the components for which the abscissa scale is enlarged 1,000 times.

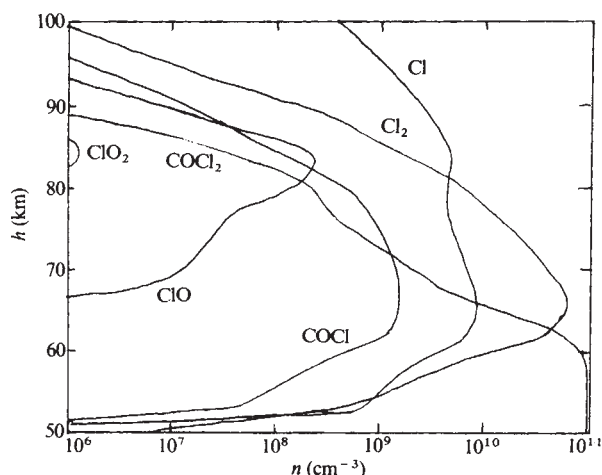


Fig. 3 Number density of chlorine compounds found in the atmosphere of Venus.

Venera spacecrafts and suggest that these measurements are correct and that H_2SO_4 formation is the basic drying agent in the above-cloud atmosphere.

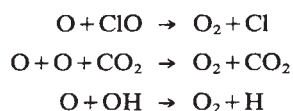
Both profiles are determined by upward transport of SO_2 and H_2O from the subcloud atmosphere and their photochemical transformation into sulphuric acid drops through the SO_2 photolysis, SO_3 formation and absorption of H_2O . Analytically derived ratios and computations of H_2O and SO_2 number densities show that the eddy mixing coefficients should be $K = 2\text{--}5 \times 10^4 \text{ cm}^2 \text{ s}^{-1}$ at 50–60 km, to ensure a good fit with the spectroscopic ground-based and satellite measurements^{16–18} of SO_2 and H_2O .

Eddy mixing determines not only H_2O and SO_2 number densities but also the amount of sulphuric acid: the higher K , the more H_2SO_4 should be in the cloud layer. The calculated amount of H_2SO_4 corresponding only to the second mode of the particle size distribution^{19,20} may be obtained for $K = 100 \text{ cm}^2 \text{ s}^{-1}$ which is not consistent with atmospheric dynamics and the observed SO_2 distribution.

A good agreement of the calculated values with the measured SO_2 number densities and the aerosol amount in the middle cloud layer may be obtained when $K = 2 \times 10^4 \text{ cm}^2 \text{ s}^{-1}$, in this case both the second and the third modes^{19,20} of particle size distribution in the middle cloud layer are dominated by sulphuric acid or the products of its further conversion (sulphates). For this conclusion, sulphuric acid must predominate drying in the above-cloud atmosphere.

Sulphur particles can constitute only a minor component of the cloud layer; their mass is an order of magnitude smaller than that of sulphuric acid and its products. The calculated concentration of sulphuric acid varies with height and is 80–87% at 55–70 km. The results of calculations are very sensitive to small variations of the $\text{H}_2\text{O}/\text{SO}_2$ ratio at the lower boundary (50 km). A 10% increase of $f_{\text{H}_2\text{O}}$ is followed by a sharp increase in humidity in the upper cloud layer, far beyond the limits of the ground-based spectroscopic measurements¹⁴ ($f_{\text{H}_2\text{O}} = 10^{-(5-7)}$ at 62–65 km), by the much larger amounts of atomic hydrogen in the upper atmosphere.

The main paths of the formation of O_2 from atomic oxygen produced by CO_2 photolysis are the reactions



Its destruction occurs through



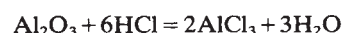
O_2 forms a layer 15-km thick at 90 km. The total amount of O_2 is $3.7 \times 10^{19} \text{ cm}^{-2}$ which is a factor of 1.5 lower than the spectroscopic limit²¹. It is inversely proportional to K , hence $K \leq$

$6 \times 10^5 \text{ cm}^2 \text{ s}^{-1}$ at 90 km. The above-cloud atmosphere cannot be a source of O_2 in the cloud layer where $f_{\text{O}_2} \approx 4 \times 10^{-5}$, according to Pioneer Venus measurements⁵. The presence of such amounts of O_2 in the clouds should lead to another order-of-magnitude reduction of the aerosol sulphur abundance but it does not contradict the spectroscopic limit as the O_2 profile will be similar to the profiles of SO_2 and H_2O . However, this does not explain the appearance of O_2 in the cloud layer as the presence of O_2 is impossible in the lower atmosphere.

For the calculations and measurements to agree K must be $2 \times 10^4 \text{ cm}^2 \text{ s}^{-1}$ at 50–60 km, $K \leq 6 \times 10^5$ at 90 km; the data on atomic oxygen in the upper atmosphere impose $K = 4 \times 10^6$ at $Z > 110$ km. At heights of 60–110 km the values obtained are well described by the dependence $K \sim n^{-1/2}$ which agrees well with the Pioneer Venus results¹⁴ and was assumed in our calculations.

The photochemistry of the atmosphere of Venus at 58–96 km was considered recently by Winick and Stewart¹¹. The main difference in input data between their model and ours is the absence of some chemical processes (53 instead of our 102 reactions); the most important of these are given in Table 2. The other difference is the assumption of height-independent H_2O mixing ratio $f_{\text{H}_2\text{O}} = 10^{-6}$ instead of $f_{\text{H}_2\text{O}} = 2 \times 10^{-4}$ at the lower boundary in our calculations, which shows very non-uniform distribution of H_2O (Fig. 2). Number densities of CO , O_2 , O_3 , SO calculated by Winick and Stewart¹¹ exceed the results of spectroscopic observations^{8,21–23}.

The X-ray fluorescence analysis²⁴ of the cloud-layer particles of Venera 11 and 12 indicated the presence of considerable amounts of chlorine ($\sim 2 \times 10^{-9} \text{ g cm}^{-3}$) in the aerosol. The presence of HCl drops is impossible because of the presence of sulphuric acid, and AlCl_3 is the most probable chlorine bearing material. If the Al_2O_3 abundance in the Venus lithosphere is similar to its abundance on Earth (15% in sediments), then the presence of HCl in the atmosphere implies a certain presence of aluminium chloride due to the heterogeneous thermochemical reaction



If its mixing ratio is 10^{-4} , it should be condensing at the lower cloud layer, and the calculated parameters of the layer⁷ (thickness, 2 km; $\rho_{\text{max}} = 4 \times 10^{-7} \text{ g cm}^{-3}$ for $K = 2 \times 10^4 \text{ cm}^2 \text{ s}^{-1}$; size particle, 5 μm) do not differ much from those measured¹⁹.

Thus the third particle mode may have different composition in the middle and lower cloud layers. This is not a contradiction, because the nephelometer measurements²⁰ show that the middle cloud layer is very uniform and may have a photochemical nature while the lower cloud layer is variable and has a condensation origin.

The presence of chlorides in the cloud layer has recently been confirmed. The atmospheric albedo in the near UV is explained by the presence of two absorbers¹⁸: SO_2 and 1% FeCl_3 solution in the concentrated sulphuric acid. FeCl_3 condensation probably occurs near the lower cloud layer and the fine-fraction of the condensate with sizes of $\sim 0.5 \mu\text{m}$ moves through mixing in the upper cloud layer to act as sites of H_2SO_4 condensation. With

Table 2 Reactions of COCl and COCl_2 in cycles of O_2 destruction and CO_2 formation

No.	Reaction	Rate coefficient ($\text{cm}^3 \text{ s}^{-1}$ and $\text{cm}^6 \text{ s}^{-1}$ for two- and three- body reactions)
(1)	$\text{CO} + \text{Cl} + \text{CO}_2 \rightarrow \text{COCl} + \text{CO}_2$	3×10^{-33}
(2)	$\text{COCl} + \text{O}_2 \rightarrow \text{CO}_2 + \text{ClO}$	2×10^{-14}
(3)	$\text{COCl} + \text{O} \rightarrow \text{CO}_2 + \text{Cl}$	10^{-13}
(4)	$\text{COCl} + \text{Cl}_2 \rightarrow \text{COCl}_2 + \text{Cl}$	$6 \times 10^{-13} \exp(-1,400/T)$
(5)	$\text{COCl} + \text{Cl} \rightarrow \text{CO} + \text{Cl}_2$	$6 \times 10^{-14} \exp(-400/T)$
(6)	$\text{COCl} + \text{CO}_2 \rightarrow \text{CO} + \text{Cl} + \text{CO}_2$	$10^{-12} \exp(-3,200/T)$
(7)	$\text{COCl} + \text{H} \rightarrow \text{CO} + \text{HCl}$	10^{-13}
(8)	$\text{COCl}_2 + \text{O} \rightarrow \text{CO}_2 + \text{Cl}_2$	10^{-14}

this size ratio the resulting FeCl_3 solution concentration is close to 1%.

Thus aerosol particles in the upper cloud layer consist of drops of 80–85% sulphuric acid and a small amount of FeCl_3 (~1%). In the middle cloud layer the major components are sulphuric acid and its chemical conversion products such as sulphates.

The presence of nitrosylsulphuric acid²⁵ seems doubtful due to the smaller amount of lightning²⁶ and its production of NO without further conversion to NO_2 , which needs large amounts

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of HO_2 and is necessary for nitrosylsulphuric acid formation. The lower cloud layer most probably consists of aluminium and iron chlorides. The presence of ammonium chloride is less likely as thermochemical calculations preclude the possibility of ammonia appearance in the lower atmosphere, and the photochemical formation of ammonia is highly unlikely.

Note added in proof: Further data on the temperature and position of the lower cloud layer preclude AlCl_3 being its main component.

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Radiation-enhanced exhalation of hydrogen out of stainless steel

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The state and diffusion of hydrogen in solids have been studied extensively^{1,2}, and the transport of hydrogen and its isotopes in metals has been found to involve classical diffusion and quantum effects such as tunnelling migration and small polaron hopping^{3,4}. The permeability and diffusion of hydrogen isotopes, particularly tritium (T), have been studied in a D-T fusion device⁵. Diffusion of tritium in the first wall of fusion reactor is expected to occur under high radiation fields due to plasma radiation, self β rays from the held-up tritium and neutron-induced radioactivity⁶. Heinrich *et al.* observed α -ray enhanced hydrogen permeation in an iron foil due to activated adsorption of the ionized hydrogen gas on the metal surface⁷. The γ -ray irradiation from a 1 mCi source, however, caused no appreciable change in permeation in 316 stainless steel⁸. No detectable loss of tritium was observed from a niobium sample containing 1% tritium⁹: the effect of the self β rays on tritium diffusion in niobium seems to be negative. We report here an experimental study of radiation-enhanced diffusion of hydrogen in hydrogen-charged stainless steel using direct observation of hydrogen exhalation and lattice dilatation.

Hydrogen was charged into type 304 stainless steel of thickness ~100 μm by cathodic charging in 0.5 M H_2SO_4 solution at a current density of 30 mA cm^{-2} . Exhalation of gaseous hydrogen was detected using volume measurement of hydrogen gas bubbles by immersing the specimen in liquid glycerine and monitoring the increase of the liquid level in a glass microcylinder. Hydrogen bubbles on the surface were examined microscopically by coating the specimen with glycerine and hydrogen-induced lattice expansion was studied by X-ray diffraction. To enhance hydrogen evolution the X-ray tube was operated at 45 kV and 15 mA. The exposure rate of $30 \pm 5 \text{ rad s}^{-1}$ was determined with thermoluminescence dosimeters (TLD) of CaSO_4 taking the energy dependence of TLD into account. In some experiments γ -irradiation from a ^{60}Co source was also used.

Figure 1 shows the enhancement by X-ray irradiation of the gas exhalation rate out of stainless steel as a function of the time after 30 min of hydrogen charging. Exhalation rates, both with

and without irradiation, decreased gradually with the time but the decrease was not monotonic presumably because of non-uniform distribution of hydrogen in the specimen. The exhalation rate of $4 \times 10^{-6} \text{ cm}^3 \text{ cm}^{-2} \text{ s}^{-1}$ at 10 min is enhanced to $6 \times 10^{-6} \text{ cm}^3 \text{ cm}^{-2} \text{ s}^{-1}$ by X-ray irradiation. The exhalation rate of hydrogen molecules deduced from the hydrogen gas volume exhalation rate is shown on the right coordinate of Fig. 1. The total volume of hydrogen gas exhaled was $\sim 3 \times 10^{-2} \text{ cm}^3 \text{ cm}^{-2}$ or $8 \times 10^{17} \text{ H}_2 \text{ cm}^{-2}$ indicating an initial average concentration of $\sim 1.6 \times 10^{20} \text{ H atom cm}^{-3}$ for a specimen ~100 μm in thickness—a number roughly two orders of magnitude smaller than that of f.c.c. unit cells.

Part of the hydrogen-charged specimen was covered with glycerine after the hydrogen exhalation had ceased and was exposed to X rays at room temperature. Hydrogen bubbles were observed on the surface of the specimen at the irradiated site as shown in Fig. 2a. No hydrogen bubble was detected at the non-irradiated site at this stage (Fig. 1b). The temperature of the same specimen was then raised to ~80 °C and new bubbles formed only at the non-irradiated site. Hydrogen atoms trapped and stabilized in stainless steel would have been released by X-ray irradiation.

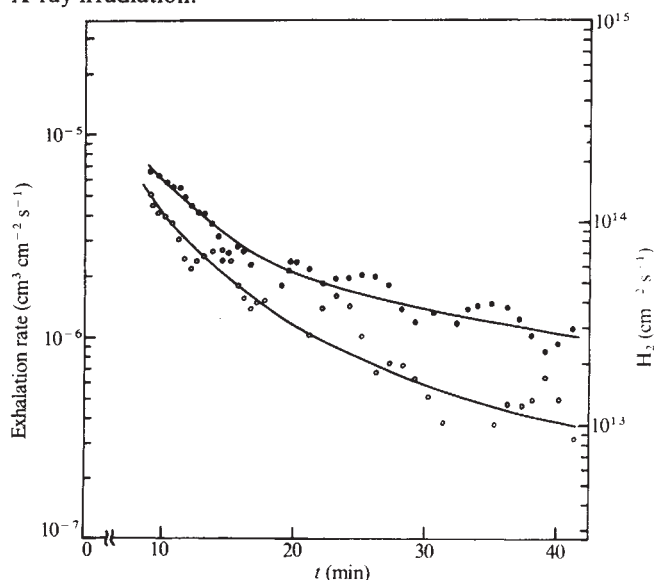


Fig. 1 The exhalation rate of hydrogen gas out of stainless steel 304 in liquid glycerine under X-ray irradiation (●) and without irradiation (○) as a function of time after the cathodic hydrogen charging in 30 min. The exhalation rate is enhanced by the radiation.

Hydrogen bubbles were not uniformly distributed on the specimen surface and their diameters were also random. The exhalation behaviour was different during irradiation; tiny bubbles appeared all over the surface and formed a 'film' of hydrogen gas on the surface. Presumably, exhalation from the grain surface by radiation-induced bulk diffusion dominates over grain boundary exhalation. No hydrogen bubble formed during X-ray irradiation if the stainless steel was not cathodically charged with hydrogen; confirming that the bubbles were not due to radiolysis of glycerine.

Enhancement of the exhalation rate of $2 \times 10^{-6} \text{ cm}^3 \text{ cm}^{-2} \text{ s}^{-1}$ or $5.4 \times 10^{13} \text{ H}_2 \text{ cm}^{-2} \text{ s}^{-1}$ on exposure to X rays at 30 rad s^{-1} indicates that $\sim 1.8 \times 10^{12} \text{ H}_2$ molecules are exhaled by 1 rad or $5 \times 10^{13} \text{ eV g}^{-1}$. In terms of hydrogen atoms exhaled, $3.6 \times 10^{12} \text{ H cm}^{-2} \text{ rad}^{-1}$ is greater than the number of the ion pairs produced by 1 rad in 0.03 cm^3 gaseous hydrogen. The radiation energy absorbed by the $100\text{-}\mu\text{m}$ thick stainless steel of density 8 g cm^{-3} is roughly $4.0 \times 10^{12} \text{ eV}$ for exposure to 1 rad. Thus, for one hydrogen atom exhalation the input energy was $\sim 1 \text{ eV}$. The efficiency decreases so that $\sim 4 \text{ eV}$ per hydrogen atom exhalation is required 40 min after hydrogen charging. It is assumed that the probability of re trapping before hydrogen diffuses to the surface increases as the concentration of hydrogen is decreased. If the hydrogen concentration is high, the traps are almost all occupied by hydrogen. Most energy would be used to ionize the host stainless steel lattice atoms. The net energy absorbed at the unit cells with a hydrogen atom would contribute to causing the release of hydrogen in a cluster model, if the energy transfer process is absent. Thus, around 10^{-2} eV is required to detrapp a hydrogen atom. It is not known how many re trapping and detrapping events are occurring, however, considering the high concentration, it seems that hydrogen comes out of stainless steel without an appreciable number of re trapping events. A similar radiation-enhanced exhalation rate of hydrogen out of stainless steel was observed on measuring pressure change with the specimen in an evacuated cell.

The lattice constant of a metal is increased by the dissolution of hydrogen. Figure 3 shows X-ray enhanced recovery of the lattice expansion measured from the shift of X-ray diffraction peaks for thin foils of the stainless steel with the thickness of

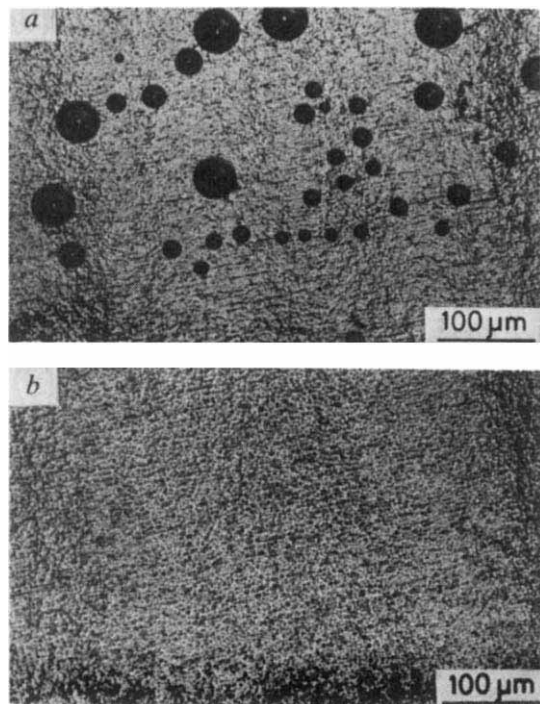


Fig. 2 Surface micrographs of electrolytically hydrogen charged stainless steel 304 coated with glycerine after 3.4 h ageing following the light charging for 5 min. *a*, Hydrogen bubbles induced at the irradiated site; *b*, no bubble was observed at the non-irradiated site.

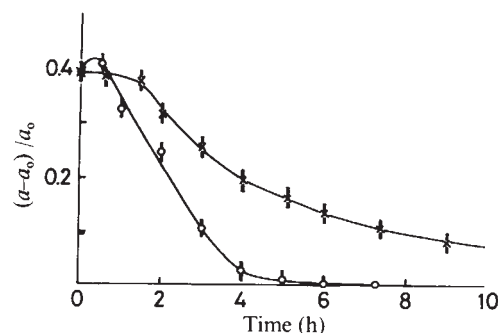


Fig. 3 X-ray enhanced recovery of lattice expansion induced by hydrogen in thin stainless steel foil. Fractional changes of the lattice constant determined from the X-ray diffraction peak are shown as a function of the time of X-ray irradiation (O) and of the ageing time for the non-irradiated specimen (x).

$\sim 35 \mu\text{m}$. The lattice expansion of $\sim 0.4\%$ was decreased to 0.2% in $\sim 4 \text{ h}$ and recovered in 12 h . On the other hand, it was reduced to 1.5 h for the fractional expansion to be the half and to 4.5 h for the complete recovery under X-irradiation. No hydrogen bubble was detected from the sample coated with glycerine after X-ray irradiation for 4.5 h although bubbles were still observed at the non-irradiated sample in agreement with the observation of the lattice expansion.

The lattice expansion persisted at 77 K without the X-ray irradiation for a long time. The X-ray or γ -ray irradiation at 77 K decreased the expansion and restored the lattice parameters. The result indicates that the hydrogen exhalation operates in the region where the monitoring X rays penetrate for the diffraction studies. The exhalation would not be caused by the radiation-induced decrease of the surface impedance at the surface barrier layer because the lowering of the surface impedance would not lead to the exhalation of hydrogen at 77 K where hydrogen is not mobile without irradiation. It also seems unlikely that specimens dipped in liquid nitrogen are heated by radiation. Thus, we conclude that the radiation has induced hydrogen diffusion in the bulk of the specimen. The present observation contrasts to that of Heinrich *et al.*, in which surface impedance from gaseous hydrogen to the iron was found to be reduced by the α -ray ionization of hydrogen as in a permeation study⁷.

It is still premature to assign the radiation-induced hydrogen diffusion a definite mechanism, but two possible mechanisms can be suggested tentatively. The electronic structure of hydrogen in metals has recently been calculated using a cluster molecule model involving the nearby metal electron orbitals⁹. Hydrogen in metals also forms a stable quasimolecule, a diatomic complex, with C, N and O impurity¹⁰ and with Ni in Fe-Ni alloys¹¹. Ionizing radiation might excite such quasimolecules or cluster molecules to antibonding or non-bonding states—such states were reported $1\text{--}2 \text{ eV}$ above the valance band in studies of photoelectron emission and soft X-ray spectroscopy^{12,13}, and can be demonstrated by theoretical calculations⁹. Decomposition of the cluster might follow when antibonding or non-bonding states are reached. If this is the case, the input energy of $\sim 1 \text{ eV}$ per H atom exhalation was used effectively by some energy transfer process.

Destruction of the cluster or diatomic complex may also be explained by the core-hole Auger decay model for electron- or photon-induced desorption of gas molecules on the surface of solid¹⁴. However, the contribution of Auger process would be small considering the high efficiency of $\sim 1 \text{ eV}$ per hydrogen exhalation, and the model of cluster excitations remains the more likely explanation.

Energy transfer to hydrogen or to the proton by elastic collisions with photoelectrons and Compton electrons is also plausible. Like the electrotransport and heat transport mechanism for hydrogen migration², electron scattering at hydrogen causes a momentum transfer from electrons to hydrogen. No work has yet been done to prove the possibility of this

mechanism. However, it is conceivable that hydrogen is detrapped by the transfer of $\sim 0.1\text{--}0.01\text{ eV}$ of energy from electrons by collisions.

The permeation rate was not affected appreciably by γ -rays from a 1 mCi source⁵. The dose rate in that case, assuming an average distance of 1 cm from the source, is only 10 rad h^{-1} , four orders of magnitude smaller than 10^5 rad h^{-1} in the present experiment. The 'hold-up' of tritium in the first wall of a fusion device is a severe problem. The hydride at the surface wall would be destroyed by radiation of the order of $10^6\text{--}10^8\text{ rad h}^{-1}$ and also by the X-ray flux from the plasma. The reduction of tritium diffusion due to the blocking by transmutation-produced ^3He (ref. 15) and to the trapping by defects or impurities might be compensated by the radiation-induced tritium diffusion. Further investigations are necessary to explain this radiation-induced hydrogen exhalation. Details of the X-ray diffraction studies will be published elsewhere with the result of isotope effects.

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Expected immersion of Saturn's magnetosphere in the jovian magnetic tail

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Voyager 2 approaches Saturn (τ) this month, August 1981, after the possible encounter^{1,2} of the planet with the tail or wake³ of Jupiter ($\mathcal{J}$). With the magnetic flux in the tail of $\phi \approx 2 \times 10^{12}\text{ Wb}$ (refs 4, 5) a simple model suggests that the tail is very long (7–15 AU) and wide enough ($\sim 0.6\text{ AU}$) to engulf Saturn. This could result in a sudden drop (by a factor of ~ 40) of the ram pressure on the magnetosphere of Saturn. The ensuing inflation of the magnetosphere may cause effects observable from Voyager 2 and/or Earth-orbiting satellites, including a flare-up of kilometric radiation and enhancement of the Ly α limb brightening. Such events, if observed, could shed light on the magnetic and plasma nature of the jovian tail and on the electrodynamics of the saturnian magnetosphere.

The expected length of the jovian magnetic tail is^{6–8} approximately

$$L_t = 2B_1 r_{pc}^2 / (\alpha B_s y_c) \quad (1)$$

where $2y_c$ is the width of the jovian magnetosphere at $x=0$ (centre of the planet, see Fig. 1 inset), B_1 , B_s are the ionospheric and the solar wind magnetic field intensities respectively, r_{pc} is the radius of the polar cap, and $\alpha < 1$ parameterizes the efficiency of reconnection⁷. Taking $y_c = 87 R_J$ (where R_J is the radius of Jupiter⁹), $B_1 = 1\text{ mT}$ and $r_{pc} = 0.28 R_J$ (Voyager data^{4,5,10,11}), $B_s = 0.6\text{ nT}$ (ref. 12) and assuming $\alpha = 0.1\text{--}0.2$ (as for Earth⁸) one obtains $L_t = 7\text{--}15\text{ AU}$. Thus, the jovian tail could be several times longer than the Jupiter–Saturn distance $x_s \approx 4.3\text{ AU}$.

Orbital considerations suggest that Saturn should miss the axis of the jovian tail by $\Delta \approx 380 R_J$. This event (for a 400–

450 km s^{-1} solar wind) would have been expected during the first week of May 1981. Immersion requires $\Delta < y(x_s)$, the half-width of the tail at Saturn's orbit (see Fig. 1 inset).

Recent Voyager 1 and 2 Jupiter flybys identified a tailward-oriented magnetic field (3–5 nT at $x_0 = 80\text{--}180 R_J$ (refs 5, 10, 11)) and two populations of circumjovian plasma: a cold one^{13–16} (densities¹³ $n \sim 10^{-5}\text{ cm}^{-3}$) on the pre-dawn tail-like region of the magnetosphere, and a (denser, $n \sim 10^{-4}\text{--}10^{-3}\text{ cm}^{-3}$) hot plasma beyond $x \sim 160 R_J$ streaming nearly antisunward^{17–19}. Based on these data we have constructed a simple magnetohydrodynamical, one-dimensional model of the distant jovian tail.

We assume that the tail shape $y(x)$ is determined by the condition of tail pressure equalizing total solar wind pressure

$$B_t^2/8\pi + p_t = m_s n_s v_s^2 \cos^2 \psi + p_s + (\mathbf{B}_s \cdot \hat{\mathbf{e}})^2/8\pi \quad (2)$$

where $\hat{\mathbf{e}}$ is a unit vector ($\hat{\mathbf{e}} \perp \hat{\mathbf{n}}$ or $\hat{\mathbf{e}} \parallel \mathbf{B}_s$, where $\hat{\mathbf{n}}$ represents a unit outward vector normal to the tail boundary $y(x)$, Fig. 1 inset).

The tail pressure consists of two components: magnetic $B_t^2/8\pi$ with the planetary magnetic field B_t (and the plasma streams) parallel to the tail boundary $y(x)$, and particle thermal pressure $p_t = 2n_t kT_t$. Conservation is also assumed of the magnetic flux, the mass flux, and the momentum

$$B_t y^2 = \text{constant}, \quad n_t v_t y^2 = \text{constant}, \quad m_t n_t v_t dv_t = -dp_t \quad (3)$$

of the jovian plasma streaming away along the tail with bulk velocity v_t . This plasma is assumed to be adiabatic and either anisotropic (CGL-approximation: $p_t^{\parallel} \propto n_t^2/B_t^2$, $p_t^{\perp} \propto n_t B_t$) or isotropic $p_t^{\parallel} = p_t^{\perp} = p_t \propto n_t^{5/3}$. All quantities: y , v , n , T , p and B are functions of x ; m denotes effective ion mass (pure H and pure O were tried, see ref. 17).

The solar wind magnetic field pressure is due either to the tangential component only ($\hat{\mathbf{e}} \perp \hat{\mathbf{n}}$), which seems appropriate for $y(x)$ in the ecliptic plane, or to the full field ($\hat{\mathbf{e}} \parallel \mathbf{B}_s$) which applies in the solar meridian plane. A standard Archimedean spiral model was used for $\mathbf{B}_s$ (ref. 12) with a radial ($B_r \propto r^{-2}$, $r^2 = (x+d)^2 + y^2$, see Fig. 1 inset) and an azimuthal component ($B_\phi \propto r^{-1}$). At Jupiter: $B_r = 0.08\text{ nT}$, $B_\phi = 0.58\text{ nT}$. The solar wind is spherically symmetric with the thermal pressure $p_s = 2n_s kT_s$, ($n_s \propto r^{-2}$, $n_s(2\mathcal{J}) = 0.28\text{ cm}^{-3}$, $T_s = \text{constant} \approx 0.25 \times 10^5\text{ K}$ (ref. 12) and the ram pressure equal to $m_s n_s (\mathbf{v}_s \cdot \hat{\mathbf{n}})^2$, where $v_s = \text{constant} \approx 450\text{ km s}^{-1}$ (ref. 12). Both the magnetic (tangential) pressure and the ram pressure depend on the angle of attack (defined in Fig. 1 inset: $\cos \psi = -\hat{\mathbf{v}}_s \cdot \hat{\mathbf{n}}$) which is related to the tail shape $y(x)$ by the equation

$$dy/dx = \cotan(\psi - \varphi), \quad \tan \varphi = y/(x+d) \quad (4)$$

Equations (2)–(4) were numerically integrated for several cases given in Fig. 1 and Table 1, yielding y, v, n, T, p, B in the tail. The

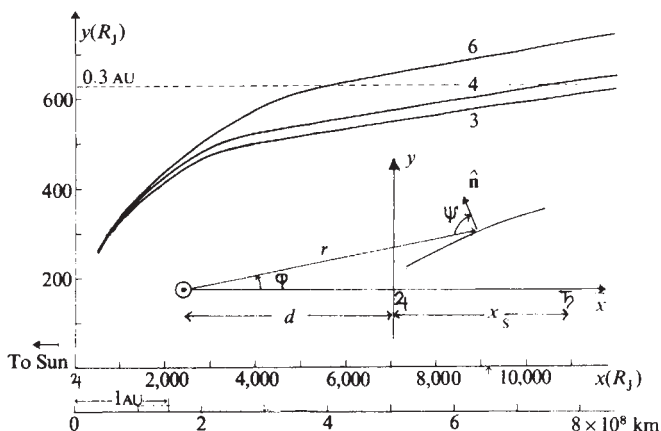


Fig. 1 Predicted boundary of Jupiter's distant magnetic tail in the jovicentric rectangular frame. The scale of ordinates is 10 times larger than the scale of abscissae. The shape of the tail was found by numerical integration of the pressure balance equation (2) for several cases, as shown in Table 1 and explained in the text. Assumed geometry is schematically shown in the inset. The most probable tail width (curve 4) at Saturn $2y_s = 1,212 R_J = 0.58\text{ AU}$.

Table 1 Predicted jovian magnetic tail values at Saturn

Ram*	Solar wind pressure		Tail pressure		at Saturn [†] ($x = x_s$)			
	Thermal	Magnetic	Magnetic	Thermal	$y_s = y(x_s)$ (R_J)	nv ($\text{cm}^{-2}\text{s}^{-1}$)	mnv^2 (erg cm^{-3})	$p = 2nkT$ (erg cm^{-3})
		Full	Tangential	Cold [‡]	Hot [‡]			
1	on [†]	off	off	on	off	980	—	—
2	on	on	off	on	off	660	—	—
3	on	on	on	on	—	579	1.8	9.9×10^{-18}
4	on	on	on	on	on	606 [§]	8.1×10^3	7.5×10^{-12}
5	on	on	—	on	—	659	1.4	7.7×10^{-18}
6	on	on	—	on	on	693	6.2×10^3	5.8×10^{-12}

$x_s = 9,170 R_J$ (1 $R_J = 71,398$ km): half-width y_s of the tail (in R_J , 1 AU = 2,095 R_J), tail plasma flux $n_i v_i$, tail wind momentum flux $m_i n_i v_i^2$ (m_i = proton mass), tail thermal pressure $2n_i kT_i$, for various terms in equation (2), 'on' or 'off'.

* For a long tail in a radial flow the ram pressure contribution vanishes for $x > x_r \sim 3,500 R_J$ ($\psi(x_r) = \pi/2$).

[†] The approximation $\phi \rightarrow 0$ in equation (4) (local direction of the solar wind parallel to the Sun-Jupiter ($\odot$ -J) line) allows an analytical solution $y^3 - y_c^3 = 2.73x(x/2 + d)$ (x, y in R_J , $d = 10,900 R_J$) leading to $y_s = 730 R_J$; this approximation is, however, not applicable to a wide tail.

[‡] For the cold plasma¹³ (few eV, $n_{i0} \sim 10^{-5} \text{ cm}^{-3}$) we assume the thermal to magnetic field energy density ratio β_0 to be $\sim 10^{-7}$ - 10^{-6} . The hot¹⁷ plasma (~ 30 keV and $n_{i0} \sim 3 \times 10^{-4} \text{ cm}^{-3}$) has $\beta_0 \sim 0.3$ (at $x_0 = 184 R_J$ and $y_0 = 181 R_J$, Voyager 1 and 2 data^{5,10,13,17}).

[§] For anisotropic plasma (CGL) one obtains a slightly lower value $y_s = 603 R_J$.

^{||} For $\beta_0 = 1$ one obtains $y_s = 772 R_J$, suggesting that for very hot plasma ($\beta > 1$) supported by more recent Voyager data²⁶, the tail should be considerably wider.

^{††} Adopted solar wind values at Saturn: $n_s v_s = 3.7 \times 10^6 \text{ cm}^{-2} \text{ s}^{-1}$, $m_s n_s v_s^2 = 2.8 \times 10^{-10} \text{ erg cm}^{-3}$, $p_s = 2n_s kT_s = 5.7 \times 10^{-13} \text{ erg cm}^{-3}$ (ref. 12).

integrations started close to Jupiter, at $x_0 = 50$ - $200 R_J$ with $y_0 = 150$ - $200 R_J$ (refs 5, 10). We assumed the plasma initially (at $x = x_0$) always isotropic with $v_i(x_0)$ equal to the local sound velocity.

Our calculations show that the tail width $2y_s$ at Saturn is large, of the order of 0.6 AU. Practically, this result is sensitive neither to the initial data nor to the details of the assumed model (see Table 1 and Fig. 1) and we suggest that Voyager 2 and Saturn will not miss the jovian tail. These unique events may shed light on several questions.

Penetration by Voyager 2 into the jovian tail may clarify two aspects of the gradual loss of identity by the tail: (1) merging of the tail and the interplanetary magnetic fields when the ratio $\mathcal{R}$ of field polarity reversal time scale to the time scale of the solar wind flow past the tail is 10^{-3} - 10^{-2} , in contrast to the terrestrial case when $\mathcal{R} \geq 1$; the spatial scale of the inhomogeneities of the plasma in the tail should be of the order of $\mathcal{R}y_s$; and (2) filling-up of the tail with the solar wind plasma through the open field lines and/or diffusion across the field. These processes should be well in progress at Saturn's distance although still far from completion²⁷. Thus information on the relative roles of these two aspects could be available well before NASA's OPEN programme²⁰.

Inside the tail, Saturn will encounter unusual conditions: a tail wind rather than a solar wind. The total pressure ($nmv^2 + p$) may drop by a factor of 30-50 and the plasma flux (nv) by a factor of 400-600 for the hot tail plasma (cases 4 and 6). This will lead to (3) a weakening or disappearance of the bow shock¹ and to (4) an inflation of the saturnian magnetosphere by a factor of ~ 2 . The inflation could be much stronger for the cold tail plasma (this seems, however, unrealistic). These effects could be directly observed by Voyager 2 if Saturn's immersion persisted till the third decade of August 1981.

Strong pressure gradients at the tail boundary crossing on a time scale of 1 - 2×10^5 s may trigger a gigantic substorm followed by (5) a flare-up of kilometric radiation²¹⁻²³ and (6) an enhancement (by particle precipitation) of Saturn's Ly α limb brightening²⁴. On the other hand, during prolonged immersion, the external magnetic field will become parallel to the plasma velocity which will lower the efficiency of magnetic field reconnection and magnetospheric dynamo.

Several observable effects are then to be expected: plasma and magnetic field effects (1), (2) (and with luck (3) and (4)) could be detected from Voyager 2; the kilometric radio wave flare (5) could be seen from Voyager 2 (and the Earth's orbit?) and the possible UV effect (6) could be detected from Voyager 2 and with some effort from the Earth's orbit²⁵.

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Extended hopanes up to C₄₀ in Thornton bitumen

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Gas-chromatographic and mass-spectrometric investigation of the saturated hydrocarbon fraction of a distillation cut of Thornton bitumen has revealed the presence of diastereomeric pairs of side-chain-extended 17 α (H)-hopanes with carbon numbers ranging up to C₄₀. Pentacyclic triterpanes of the hopane type are ubiquitous in sediments and related fossil fuels¹. Commonly, besides the parent hydrocarbon itself, 17 α (H)-hopane (C₃₀), compounds with degraded (C₂₇, C₂₉) and extended side-chains (C₃₁-C₃₅) are detected². While hopanes with 30 or fewer carbon atoms can easily be interpreted as diagenetic products of C₃₀ hopanoids, for example, hop-22(29)-ene, known in several living organisms³, the extended hopanes (C₃₁-C₃₅) have recently been related to a C₃₅ precursor, bacteriohopanetetrol, which was found to be a constituent of several microorganisms^{1,4-6}. We report here that the new C₃₆ C₄₀ hopanes are of the same structural type as the well-known extended hopanes ranging up to C₃₅.

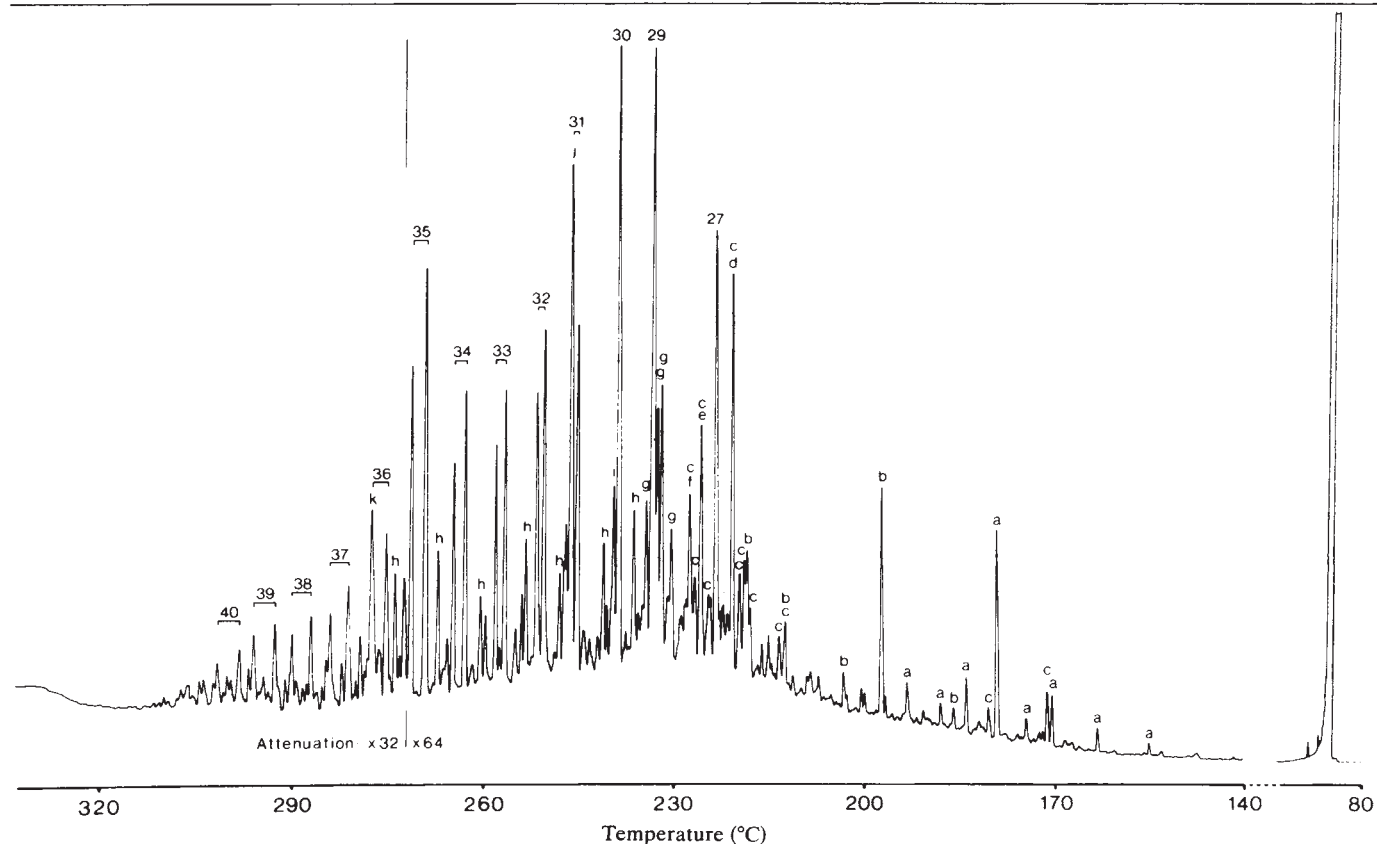
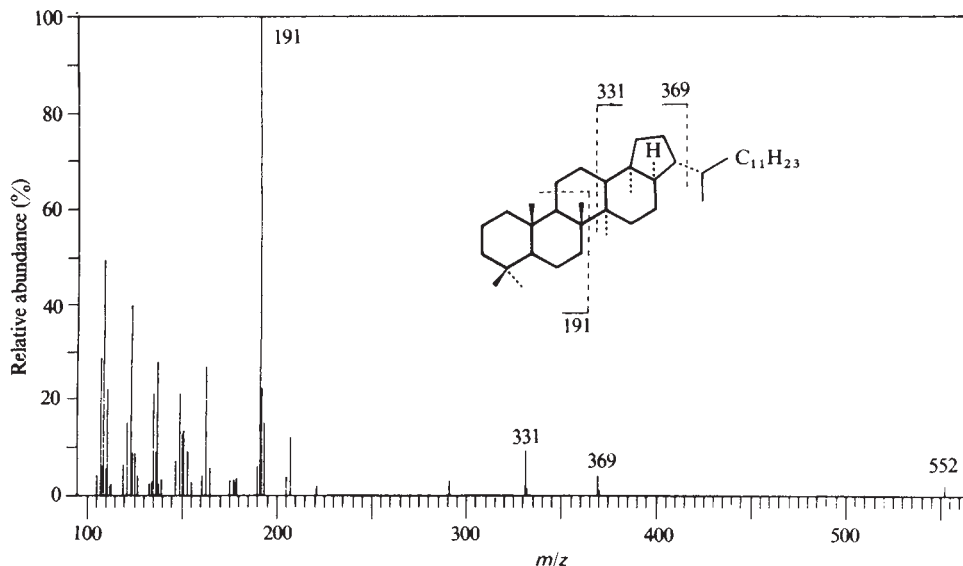


Fig. 1 Capillary column chromatogram of the saturated hydrocarbon fraction of a Thornton bitumen distillation cut. Numbers indicate 17α (H)-hopanes (carbon atoms); a, tricyclic terpanes (C_{20} – C_{25}); b, tetracyclic terpanes (C_{23} – C_{25} , C_{29}); c, steranes (C_{21} – C_{22} , C_{26} – C_{28}); d, 18α (H)-trisorneohopane; e, pentacyclic C_{28} -triterpane; f, C_{29} -sterane; g, 24-ethyl-5 α (H)-cholestanes [14α (H), 17α (H), (20S)-, 14β (H), 17β (H)(20R)-, 14β (H), 17β (H)(20S)-, and 14α (H), 17α (H)(20R)- isomers in order of elution]; h, moretanes (C_{29} – C_{35}); i, pentacyclic C_{30} -triterpane; j, gammacerane (?); k, perhydro- β -carotene (?). A Varian 3700 gas chromatograph was used with FID-detector; 25 m $\times$ 0.3 mm i.d. glass capillary column was coated with high-temperature SE 54 (Carlo Erba); injector, 300 °C; detector, 300 °C; carrier gas, helium; temperature programme, 80–330 °C (3 °C min⁻¹).

Thornton bitumen was obtained from the Thornton Quarry, just south of Chicago (Illinois). The bitumen is found in the Niagara dolomite deposited during Silurian times, and it is thought that it was formed simultaneously with the rock and thus may also be of Silurian age⁷. Rounded hillrocks of the ancient dolomite now protrude glacial clay. The rock is highly fossiliferous and contains unicellular species, and plant and animal skeletons⁷. A previous study of the saturated hydrocarbons from the bitumen by Marschner *et al.*⁷ revealed the absence of *n*-alkanes and a prominent contribution of steranes and triterpanes although no specific identifications were made.

As can be seen from the capillary column chromatogram in Fig. 1, the saturated hydrocarbon fraction of Thornton bitumen is enriched in pentacyclic triterpanes, most of which have a hopane skeleton. The most abundant compound is 17α (H)-hopane (C_{30}). 17α (H)-norhopane (C_{29}), 17α (H)-trisorneohopane (C_{27}) and 18α (H)-trisorneohopane (C_{27}) were found as major degraded hopanes. Further tetra- and tricyclic terpanes, based on their mass spectral base peaks at m/z 191, may have structures related to hopane and may, at least in part, be derived from hopanoid precursors by degradation during diagenesis and catagenesis. Smaller amounts of steranes in Thornton bitumen

Fig. 2 Electron impact mass spectrum of the first-eluting C_{40} 17α (H)-hopane (see Fig. 1). A Varian Mat 112 S mass spectrometer was used with ionizing energy 70 eV, source temperature 260 °C, gas chromatographic conditions as given for Fig. 1.



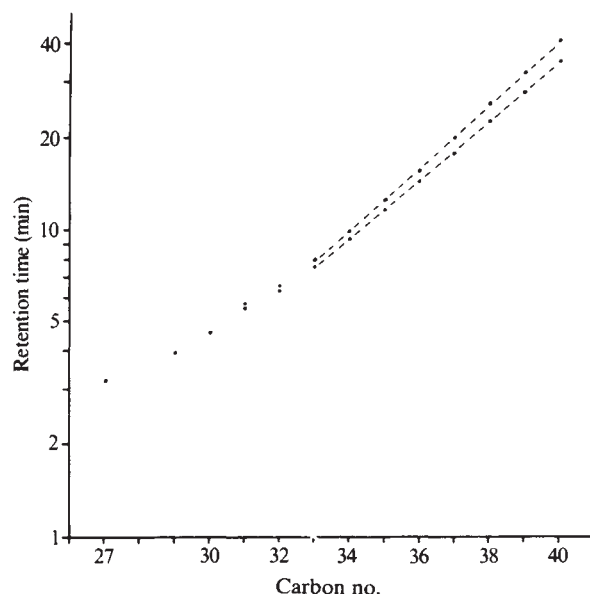


Fig. 3 Log/normal plot of retention times versus carbon number for C_{27} – C_{40} hopanes obtained from isothermal gas chromatography at 260 °C (other conditions as given in Fig. 1). The C_{33} – C_{40} hopanes show linear increase of log retention time, different for the C-22 diastereomers, indicating the presence of homologous series.

point to an advanced maturity level of the organic matter well within the oil window, as indicated by the dominance of 14β (H), 17β (H)-steranes⁸; this can easily be seen in Fig. 1 for the C_{29} steranes.

Extended 17α (H)-hopanes in Thornton bitumen are pairs of C-22 diastereomers⁹ (Fig. 1). The carbon number maximum within this series at C_{35} either indicates a particular stability of this species or, more likely, a C_{35} precursor for most of the C_{31} – C_{35} extended hopanes as mentioned above. In contrast to hopane series reported so far, the extended hopanes in Thornton bitumen range up to C_{40} . Diastereomeric pairs are observed as in the C_{31} – C_{35} range together with a slight predominance of the first-eluting diastereomer (Fig. 1; the second C_{36} isomer coelutes with a dicyclic tetraterpane, probably perhydro- β -carotene). The mass spectrum of the first-eluting C_{40} hopane in Fig. 2 shows a fragmentation pattern consistent with a hopane structure with an extended side-chain. From the log/normal plot of retention times obtained at isothermal conditions against carbon number (Fig. 3), which shows a linear increase for the C_{33} – C_{40} hopanes, the conclusion may be drawn that the side-chain extension is linear, as found for the C_{31} – C_{35} hopanes⁶, and no branching occurs.

The question now is whether the C_{40} hopanes represent another end member in this series related to a specific precursor or whether this series extends beyond C_{40} ? This cannot be unequivocally answered from the analysis of this distillation cut. No hopanes above the C_{40} members marked in Fig. 1 could be detected in this sample, which may be due to the limiting experimental conditions (temperature) or the way the distillation cut was taken (exact temperature range unknown). If there were a C_{40} precursor, the latter argument could explain the lower abundance of the C_{40} hopanes relative to the C_{36} – C_{39} compounds.

As there is no precursor yet known for the C_{40} hopanes, another mechanism for the formation of the C_{36} – C_{40} extended hopanes is suggested. It has recently been found¹⁰ that highly alkylated porphyrins in petroleum contain alkyl-chains up to C_{11} . These porphyrins seem to be catagenetic products originating from porphyrin moieties bound to the kerogen matrix and released only at higher maturation levels by thermal cracking¹¹. A similar mechanism³ for the extended hopanes seems possible if one assumes that bacteriohopanetetrol (C_{35}) is specifically

bound into the kerogen matrix during early diagenesis through a covalent carbon–carbon bond.

In addition to the extended 17α (H)-hopanes, a series of moretanes (17β (H), 21α (H)-hopanes) has been tentatively identified in Thornton bitumen (Fig. 1). The series seems to include C_{31} – C_{35} moretanes although only the C_{31} compound in the mass spectrum clearly showed the slight dominance of the D/E-ring fragment (m/z 205) over the A/B-ring fragment (m/z 191) (ref. 12). Trace amounts of these compounds have been reported¹³ for a Toarcian shale from the Paris Basin. Unlike the 17α (H)-hopanes, however, the extended moretanes were not detected as diastereomeric pairs (R and S at C-22) in Thornton bitumen; it seems possible that they are not separable in the given conditions².

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Nitrous oxide cycling in Lake Vanda, Antarctica

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Nitrous oxide (N_2O) is a key intermediate for several steps of the aquatic nitrogen cycle. In oxygenated oceanic waters, bacterial oxidation of amino- and ammonium-N is a dominant source of N_2O (ref. 1); in regions of intense nitrification ΔN_2O values (difference between observed N_2O and air-equilibrium concentration) typically rise to 40–100 nmol l⁻¹ (ref. 2). By contrast, anoxic waters are often undersaturated in N_2O because of respiratory consumption by denitrifying bacteria³. We describe here an extreme accumulation of nitrous oxide (ΔN_2O of >2,000 nmol l⁻¹) in the saline bottom waters of Lake Vanda (77°35'S, 161°40'E), a warm meromictic water body in the Dry Valley region of Antarctica. From *in situ* experiments assaying various components of the nitrogen cycle, we conclude that this N_2O is produced by a narrow band of nitrifiers lying well above the oxycline. Nitrous oxide is lost from this zone at slow rates by diffusion, ultimately to the atmosphere above, or below to the upper anoxic zone where it is consumed by a finely stratified population of denitrifying bacteria.

Lake Vanda was sampled at a mid-lake deep-water site (maximum depth 68 m) during the austral summer of 1980–81. Holes were bored through the 3–3.5-m permanent ice-cap and 1-l samples removed with a discrete-depth water sampler. The unusual temperature and salinity profiles of Lake Vanda have been described elsewhere^{4,5}. Temperatures rise with increasing depth to a warm maximum (23.5 °C) in the region of high salinity (up to three times the conductivity of seawater) at the bottom of the lake (Table 1). Over the season of study the lake was well oxygenated to 59 m (~0.7 mmol l⁻¹ throughout, S. Nakaya, unpublished data) but anoxic at 60 m and below.

Table 1 Distribution of nitrogen and other characteristics of the mid-lake water column of Lake Vanda

Depth (m)	Forms of nitrogen ($\mu\text{g-atom l}^{-1}$)					$\text{N}_2\text{O}:\text{NO}_3$ (molar ratio $\times 10^3$)	Dissolved reactive P ($\mu\text{g-atom l}^{-1}$)	Temperature ($^{\circ}\text{C}$)	Conductivity ($10^3 \mu\text{S cm}^{-1}$)
	$\text{NO}_3\text{-N}$	$\text{NO}_2\text{-N}$	$\text{N}_2\text{O-N}$	$\text{NH}_4\text{-N}$	Particulate-N				
3.25	3.5	0.10	0.05	0.9	0.7	7.1	0.02	4.5	0.6
5	4.1	0.11	—	0.6	—	—	0.01	5.0	0.7
15	2.2	0.08	0.15	0.2	0.3	34.1	0.03	6.5	1.1
25	2.1	0.04	0.18	0.1	0.7	42.8	0.01	7.0	1.2
35	2.3	0.05	0.19	0.1	0.5	41.3	0.01	7.0	1.2
45	4.4	0.02	0.51	0.7	0.4	57.9	0.02	9.0	2.3
47.5	7.3	0.07	0.72	—	—	49.3	0.02	9.5	—
50	47.0	0.17	1.96	16.9	0.6	20.9	0.01	12.5	8.0
51	89.7	0.27	2.18	19.1	—	12.1	<0.01	—	—
52	133.5	0.39	2.78	27.8	—	10.4	0.02	—	—
53	203.9	0.79	3.94	71.8	—	9.7	—	—	—
54	223.8	0.96	4.29	119.3	—	9.6	—	—	—
55	232.7	1.14	3.80	144.1	0.8	8.2	<0.01	18.5	39.5
56	165.4	1.90	3.51	269.2	—	10.6	0.02	—	—
57	106.3	0.46	2.91	363.3	—	13.7	0.04	—	—
58	56.6	0.27	2.53	458.2	1.3	22.3	0.17	20.0	56.0
59	13.2	0.33	1.04	599.4	—	39.4	0.10	—	—
60	3.1	0.10	0.05	686.9	1.9	8.1	0.15	22.0	60.9
62.5	<1.0	<0.1	<0.003	1005.6	—	—	2.21	22.5	79.5
65	<1.0	<0.1	<0.003	1346.5	—	—	5.24	23.5	80.6
67.5	<1.0	<0.1	<0.003	1747.4	—	—	8.15	23.5	80.8

Samples were collected 21 and 27 December 1980. All depths are relative to the water table, ~ 0.2 m below the ice surface. Water samples for nutrient analysis were filtered immediately on collection through acid-washed glass-fibre GF/C filters and stored frozen. Analyses were performed on a Technicon AutoAnalyzer II. Lakewater controls (sample without colour reagents) were run at all depths to correct for changes in refractive index. Further control samples were run with standard nutrient additions to correct for variable recoveries at increasing salt concentrations. Nitrite was measured by automated diazotization¹⁸. Nitrate was reduced to nitrite through a cadmium wire column¹⁹ at pH 8 (ref. 20) and then analysed by standard diazotization¹⁸. NH_4 was determined by an automated phenol–hypochlorite method²¹; high values in the bottom waters of Vanda were checked manually, after 100-fold dilution, by oxidation to nitrite and diazotization²². Dissolved reactive phosphorus was by an automated molybdenum blue method²³. Particulate nitrogen was filtered on to acid washed GF/C filters and stored frozen. Material was Kjeldahl-digested and the ammonium determined colorimetrically²⁴. For gas analysis, 15-ml lakewater samples were removed from the water sampler by hypodermic syringe and injected into 30-ml Hypovials that had been sealed with neoprene²⁵ stoppers and flushed with O_2 -free, N_2O -free nitrogen. These samples were immediately preserved with glutaraldehyde (2% v/v final concentration) and stored at 0°C for up to 5 weeks. Before analysis, the samples were shaken vigorously for 1 h to ensure equilibration between liquid and headspace. 1-ml subsamples of the gas were injected into a Perkin-Elmer Sigma 4 electron-capture gas chromatograph fitted with a 2-m (3 mm o.d.) stainless steel column of Chromosorb 102. The GC was operated at column temperature of 55°C with a carrier gas (95% Ar 5% CH_4) flow of 24 ml min^{-1} . The ^{63}Ni detector was run at 375°C with a standing current of 3.5×10^{-9} A and calibrated with standard gas mixtures. Distribution coefficients were determined later on a range of samples by multiple equilibration²⁶ to allow for the effect of increasing salinity on N_2O solubility. The original lakewater concentrations of dissolved N_2O were back-calculated²⁶ from head-space levels and the empirically determined distribution coefficients. Conductivity was measured at 2°C . The transition from oxic to anoxic water (oxycline) occurred between 59 and 60 m depth.

N_2O concentrations were well above air-equilibrium values at all depths down the oxygenated portion of the water column (Table 1). Air-equilibrium concentrations of N_2O , the saturation value for water in equilibrium with atmospheric nitrous oxide, range from 17 nmol l^{-1} at 4.5°C to 9 nmol l^{-1} at 23°C (calculated from the solubility data of Markham and Kobe⁶ and measured values for N_2O in Antarctic air⁷). Immediately beneath the ice N_2O levels were 48% above air-saturation ($\Delta\text{N}_2\text{O} = 8.2 \text{ nmol l}^{-1}$). Concentrations rose steadily with increasing depth, but sharply increased below 47.5 m to a maximum at 54 m ($>20,000\%$ air-saturation; $\Delta\text{N}_2\text{O} > 2,000 \text{ nmol l}^{-1}$). N_2O tensions rapidly declined below the peak, and the gas was undetectable at 62.5 m and below. The deep N_2O maximum closely followed a nitrate peak previously reported by Torii *et al.*⁸ and confirmed in the present study (Table 1). NO_3 and N_2O profiles are strongly correlated ($r^2 = 0.909$, $P > 0.001$) but an accumulation of nitrite in the lower depths was displaced 1–2 m downwards relative to the other forms of oxidized nitrogen (Table 1; N_2O compared with NO_2^- $r^2 = 0.606$).

At least four biological processes are thought to involve nitrous oxide⁹. N_2O is an intermediate in the oxidation of ammonia to nitrate by nitrifying bacteria:



It is also an intermediate in the reduction of nitrate to nitrogen gas by denitrifying bacteria:



Nitrous oxide has been considered a possible intermediate in assimilatory nitrate reduction by algae (reverse of the nitrifier sequence). Finally, N_2O is an alternative substrate for nitrogenase, the enzyme of N_2 fixation. This fourth process cannot be an important control on nitrous oxide levels in Lake Vanda. Acetylene reduction assays¹⁰ on lakewater samples throughout

the profile failed to demonstrate measurable nitrogenase activity at any depth.

The well-defined and coincident maxima of NO_3 and N_2O provide circumstantial evidence of a deep band of nitrifying bacteria. More conclusive evidence comes from experiments conducted *in situ* from 3.25 m to 57.5 m, to measure nitrpyrin-sensitive CO_2 fixation (Fig. 1). Significant differences ($P < 0.05$) between duplicates with and without this nitrification inhibitor were recorded only from 50 to 57.5 m. Rates were highest at 52.5 m and 55 m, in the region of abundant oxidized nitrogen.

There is some evidence from marine systems^{11,12} that nitrous oxide and nitrite may accumulate as byproducts of nitrate assimilation by algae. In Lake Vanda, as in other Dry Valley lakes¹³, algal production is low throughout most of the water column but rises to a sharp maximum just above the oxycline (Fig. 1). This photosynthetic peak lies well below the zone of N_2O accumulation, and algal assimilation is therefore unlikely to be a large source of nitrous oxide.

Denitrification has been invoked² to explain accumulation of N_2O in low-oxygen regions of the sea. Denitrification in Lake Vanda was assayed by *in situ* acetylene blockage¹⁴ experiments at depths of 55, 58, 59, 59.5, 60, 61, 62.5 and 64 m. Significant denitrifier activity was recorded only in the upper anoxic depths of 59.5–62.5 m (Fig. 1). Nitrous oxide was very low or undetectable in this region, and therefore the upper anoxic zone must be a region of N_2O consumption rather than a net source.

A comparison of the molar ratio of N_2O to NO_3 provides a further guide to the processes controlling nitrous oxide production and loss. Lowest ratios were recorded in the region 52–56 m, the zone of peak nitrifier activity. The minimum (8.2×10^{-3}) falls within the range reported for nitrifying bacteria in culture¹⁵, although it is high relative to typical accumulation ratios in the field—Elkins *et al.*¹ report an average ratio of 1.4×10^{-3} mol of N_2O produced per mol of NH_4 oxidized by aquatic nitrifiers. At higher and lower depths in Vanda the ratio

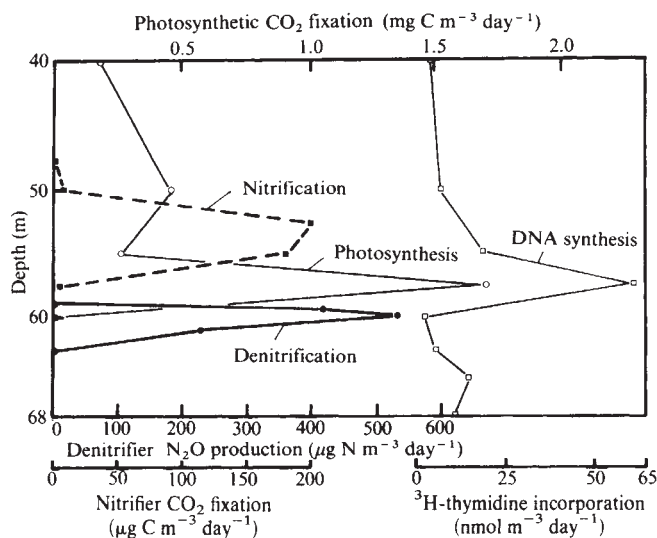


Fig. 1 Distribution of microbial activity at 40–68 m in Lake Vanda: nitrifier CO_2 fixation ($\blacksquare$), photosynthetic CO_2 fixation ($\circ$), denitrifier N_2O production ($\bullet$), ^3H -thymidine uptake ($\square$). All rates were determined by *in situ* incubations. Nitrifier activity (18 December 1980) was estimated by the difference between dark CO_2 fixation with and without the nitrification inhibitor nitrophenol²⁷. Samples were preincubated with inhibitor (10 mg l^{-1} final concentration) for 1 h at the depth of collection, and then incubated for a further 5 h with ^{14}C - HCO_3^- ($0.6 \mu\text{Ci ml}^{-1}$). Dissolved inorganic carbon was determined by IR gas analyser. Photosynthetic rates (21 December 1980) were measured by 24-h *in situ* ^{14}C - HCO_3^- ($0.6 \mu\text{Ci ml}^{-1}$) incubations in light and dark bottles. Denitrifier activity (13 January 1981) was measured by the accumulation of nitrous oxide in samples of lakewater sealed in Hypovials and incubated at the depth of collection for 24 h with 10% acetylene¹⁴, an inhibitor of nitrous oxide reductase. Samples were incubated with ^3H -thymidine (20 nmol l^{-1} , 16 December 1980) to estimate microbial rates of DNA synthesis¹⁶. At the end of the 5 h incubation, samples were filtered on to $0.22 \mu\text{m}$ Millipore membranes which were washed several times with 10% trichloroacetic acid and air-dried. The filters were later counted by liquid scintillation spectrometry and corrected for adsorption of label (glutaraldehyde-killed controls) and self-absorption of radiation.

of N_2O to NO_3^- increased, probably reflecting the selective loss of nitrate with time by algal uptake and sedimentation. Particulate nitrogen, and by inference seasonal NO_3^- removal by the plankton, is low throughout the water column (Table 1). Movement of N_2O and NO_3^- by turbulent diffusion away from the peak must therefore proceed very slowly for algal uptake to affect significantly the molar ratio of the two forms of oxidized N. This ratio decreased again from 25 m towards the ice, which would be consistent with selective loss of N_2O to the atmosphere by diffusion through the ice-cap, and at the lake edge where the ice melts away each summer to produce a moat of open water 1–10 m wide.

Denitrifier activity was not detected at depths greater than 62.5 m, where N_2O and NO_3^- were also absent: denitrification rates in Lake Vanda may ultimately be limited by the rate of transfer of oxidized N to the upper anoxic zone. Ammonium substrate is relatively abundant in the region of nitrifier activity, and it is possible that phosphorus (Table 1) rather than nitrogen availability exerts an overall control on this component of the N_2O cycle. Phosphate values increased with depth towards the oxycline (Table 1), and at these lower aerobic depths total microbial activity (as estimated by ^3H -thymidine incorporation into DNA¹⁶) increased to a water column maximum (Fig. 1). It remains unclear why the nitrifiers do not similarly peak in this superficially favourable environment at 57–59 m. A possible explanation is the steep ammonium gradient observed through this zone (Table 1). Nitrifying bacteria are inhibited by high levels of ammonia. *Nitrobacter* (nitrite oxidizer) is more sensitive than *Nitrosomonas* (ammonium oxidizer)¹⁷, and it is of interest that nitrite accumulates towards the bottom of the nitrification layer where ammonia becomes increasingly concentrated.

The relative importance of denitrifier and nitrifier activity for nitrous oxide accumulation in oceanic environments has caused considerable debate². In Lake Vanda, N_2O accumulated in the region of highest nitrifier activity where denitrification was

undetectable. Conversely, N_2O concentrations were greatly reduced in the region where *in situ* denitrification rates were maximal. In this body of water nitrification would seem to be a net source and denitrification a net sink for nitrous oxide. These different components of the N_2O cycle occupy discrete bands of the stratified water column. This layered community may thereby prove to be a useful test of future models describing nitrous oxide production and loss in natural aquatic environments.

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An opiate system in the goldfish retina

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Recently, in addition to conventional neurotransmitters such as acetylcholine, dopamine, glycine and γ -aminobutyric acid (GABA), putative neuroactive peptide transmitters have been localized to specific retinal amacrine cells¹. In particular, opiate receptors^{2,3}, assayable enkephalin immunoreactivity⁴ and enkephalin-immunoreactive neurones^{1,5} have been described in avian and mammalian retinæ. However, little physiological evidence has been obtained for the involvement of neuropeptides in retinal function. Here we report that exogenous opiates affect both the release of GABA from GABAergic amacrine cells and the firing patterns of ganglion cells in the goldfish retina⁶. Our results show that the output of the retina is modulated by an opiate system whose neural organization and pharmacological properties resemble those described elsewhere in the vertebrate central nervous system.

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Ganglion cell unit activity was recorded from the isolated, inverted retina of goldfish using metal microelectrodes. The retina was stimulated alternately at 6-s intervals for 0.7 s with a small red light spot (1 mm diameter, 650 nm, $2\mu\text{W mm}^{-2}$), or annulus (1.2 mm i.d., 3.2 mm o.d.) centred on the electrode. Ganglion cell responses were classified as ON, OFF (those responding to light with an increase or decrease of firing rate, respectively), or ON-OFF (those responding to light with bursts of spikes at onset and offset of stimulus). The retina was maintained at 22 °C in moist gas (95% O_2 + 5% CO_2), and drugs were applied to the photoreceptor side of the retina by nebulizer; no wash-out was possible. Further details are given in Fig. 1 legend.

The synthetic opioid peptide, D-Ala²-Met⁵-enkephalinamide (DALA, Peninsula), at concentrations of 0.1–1.0 μM , increased the spontaneous and light-evoked firing of 20 ON-centre units and suppressed the post-stimulus inhibition (Fig. 1a). DALA also abolished the opponent surround response to an annulus. In contrast, spontaneous and light-evoked firing in 9 of 12 OFF-centre units, was reduced by DALA; in the other three units, this inhibition was preceded by a transient period of excitation during which the cells gave ON responses to light (not shown). ON-OFF units were less predictably affected by DALA, and their activities were modified only by concentrations greater than 1.0 μM , from which they often did not recover. Morphine (0.1–1.0 μM), like DALA, excites ON-centre cells, but the excitation is prolonged (generally irreversible) and less readily modulated by light (Fig. 1b). Naloxone (4–40 μM) inhibits the dark- and light-evoked activities of ON cells, and blocks the response to DALA or morphine when applied simultaneously (Fig. 1c). Dextrorphan (4–40 μM) has no effect (not shown). These results indicate that the endogenous opioid transmitter may be released in the dark, although the possibility that naloxone could be blocking ON cell activity by acting at any synapse cannot be excluded.

To test whether DALA acts on ganglion cells directly or through intermediary neurones, all synaptic transmission to ganglion cells was first blocked by treating the retina with cobalt chloride (1–2 mM), then DALA was applied. In the presence of cobalt ions, both dark- and light-activated firing of ON-centre cells was suppressed, and the response to DALA was blocked (Fig. 1d). Thus, the receptors for DALA and morphine may be located on an intermediary neurone presynaptic to ganglion cells rather than on ganglion cells themselves. Note, however, that neuronal responses to enkephalins may require calcium currents⁷ that would be blocked by cobalt ions, thus a direct interaction is also possible.

Preliminary experiments indicated that enkephalin-immunoreactive neurites are scattered throughout the inner synaptic layer. As the dendrites of ON-centre ganglion cells and neurites of GABA-accumulating amacrine cells both ramify in the innermost sublamina of this layer⁸, the pathways of these cells were investigated further. The GABA antagonist bicuculline causes an excitation of ON-centre cells and abolition of their surround responses similar to that caused by DALA or morphine; subsequent application of DALA, however, produces no significant further increase in the firing rate (Fig. 1e). We also examined the effects of opiates on the uptake and release of GABA in the retina. We have previously shown^{8,9} that the GABA amacrine cells in goldfish can be selectively loaded by incubation with ³H-GABA in the dark, whereas type H1 cone horizontal cells are loaded by incubation in the light. Release of ³H-GABA can be induced by increasing the concentration of K^+ in the bathing medium, and the influence of opiates and their analogues on GABA release can be tested by adding these to the medium.

Dark-adapted goldfish eye cups were cut in half. The retina was isolated from each half under dim red light and incubated at 22 °C for 15 min in the dark with 0.2 ml oxygenated Ringer's solution containing 1 μM ³H-GABA (39.2 Ci mmol⁻¹, NEN). The retinæ were then incubated separately in 10 ml of unlabelled saline for 60 min with gentle shaking and a change of solution every 10 min. The efflux of ³H-GABA from each piece

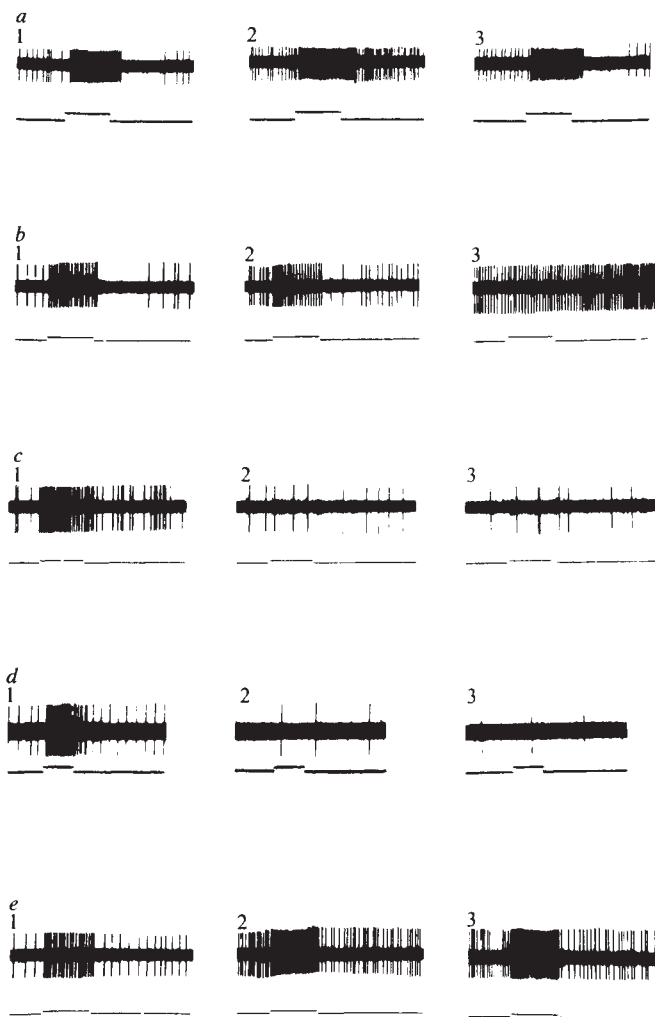


Fig. 1 Ganglion cell activity recorded extracellularly from the isolated retina of goldfish, *Carassius auratus*, placed in a chamber receptor side upwards and maintained and stimulated as described in the text. Concentrated stock solutions in cyprinid fish saline solution (120 mM NaCl, 2.5 mM KCl, 1.2 mM MgSO_4 , 2.2 mM CaCl_2 , 10 mM dextrose, 3 mM HEPES, pH 7.8) were run through an atomizer and sprayed on to the photoreceptor side of the retina; a number of solutions could be applied in turn. The dilution factor for the atomizer was estimated by spraying ³H-thymidine onto small pieces of filter paper at various dilutions and durations of application, counting in a liquid scintillation counter, and averaging the total radioactivity over a volume of retina (a circular patch 8 mm in diameter and 0.4 mm thick). Assuming that the atomized drug diffuses uniformly through the tissue, that it is neither destroyed nor taken up into retinal cells, that a steady-state concentration is achieved and that the drugs used in the physiological studies do not adhere more strongly to the stock bottles and nebulizing apparatus than ³H-thymidine, the minimum effective concentration for a readily detectable effect of DALA on ganglion cell activity was 0.1–1.0 μM . As these assumptions are certainly not valid and as each maximizes concentrations, the true minimum effective dose may be substantially lower than that estimated. The figure shows the action potentials of single ON-centre ganglion cells in the dark and in response to small, centred red light spots (square pulses below the response trace each denote 0.7 s). The pharmacological conditions were as follows: a, (1) before application of DALA (control response); (2) during maximal effect of 1.0 μM DALA; (3) 5 min after application of DALA. b, (1) Control; (2) during 1.0 μM DALA; (3) during 1.0 μM morphine sulphate, after recovery from DALA. c, (1) Control; (2) after 40 μM naloxone; (3) after 40 μM naloxone + 1 μM DALA. d, (1) Control; (2) after 1 mM CoCl_2 ; (3) after 1 mM CoCl_2 + 1 μM DALA. e, (1) Control; (2) after 10 μM bicuculline methiodide; (3) after 10 μM bicuculline methiodide + 1 μM DALA. Results shown in a–e are from different ganglion cells; 1, 2, 3 in each row show responses of the same unit.

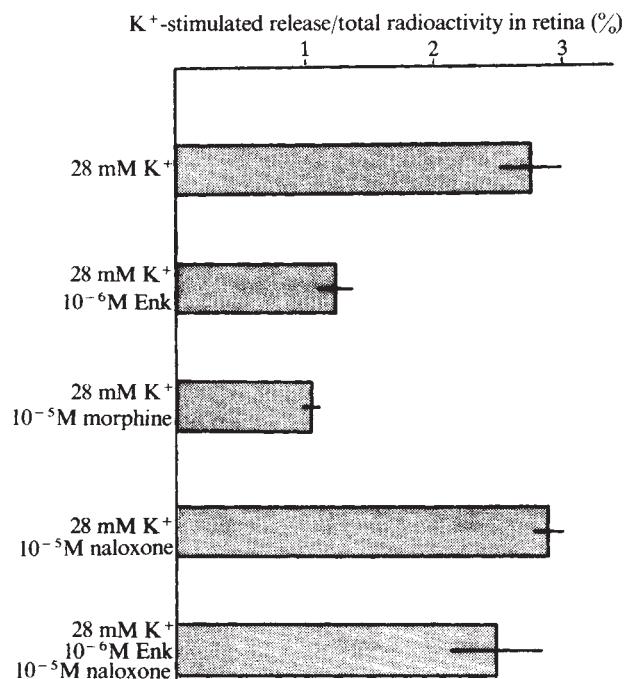


Fig. 2 The effects of enkephalin (Enk), morphine and naloxone on K⁺-stimulated release of ³H-GABA from amacrine cells of the goldfish retina. The GABAergic amacrine cells were pre-loaded by incubating the retinae in the dark for 15 min in saline containing ³H-GABA. Then the retinae were incubated with unlabelled saline for 60 min and the release of ³H-GABA into the solution was measured by scintillation counting^{13,14} every 3 min until it became constant. Each piece of retina was then transferred to K⁺-rich Ringer's solution (28 mM K⁺) to which was added Met⁵- or Leu⁵-enkephalin (10⁻⁶ M), morphine (10⁻⁵ M) or naloxone (10⁻⁵ M), alone or in combination. After incubation for 6 min (2 × 3 min) in these solutions, each retina was again transferred to normal unlabelled saline. The percentage of ³H-GABA released from the retina by K⁺-stimulation was calculated using the following formula: K⁺-induced ³H-GABA release (d.p.m.) ÷ ³H-GABA efflux in normal Ringer's solution (d.p.m.) ÷ ³H-GABA remaining in the retina (d.p.m.). Each value is the mean ± s.e.m. of at least seven experiments.

of retina was measured using methods described previously^{10,11}, except that the K⁺-rich saline contained only 28 mM instead of 56 mM K⁺, and while one half was incubated in saline, the other half was incubated in saline containing enkephalin, morphine or naloxone, alone or in combination (for details see Fig. 2 legend). Using this method, we have previously shown¹⁰ that ³H-GABA accumulated by goldfish retinal cells is released in response to high external K⁺ concentrations and that this release is inhibited by 1 or 10 mM Co²⁺ in the medium. Figure 2 shows that enkephalin and morphine suppress the K⁺-stimulated, Ca²⁺-dependent release of ³H-GABA from goldfish GABA amacrine (loaded in dark) but not from H1 cone horizontal cells (loaded in light; not shown). Both Met⁵- and Leu⁵-enkephalin have similar dose-dependent effects on GABA release, the inhibition of which ranges from 60% to 0% at 10⁻⁶ and 10⁻¹⁰ M enkephalin, respectively. Naloxone blocks the effect of enkephalin on GABA release from the amacrine cells but has no effect by itself in these incubation conditions. Furthermore, the suppression of K⁺-stimulated ³H-GABA release by enkephalin is not due to inhibition of the GABA uptake system, as retinae accumulate the same amount of ³H-GABA in the presence or absence of 10⁻⁵ M enkephalin, and enkephalin inhibits ³H-GABA release from amacrine cells even in the presence of 10⁻⁴ M nipecotic acid, a specific inhibitor of GABA uptake (Chin and Lam, in preparation).

Our results show that in the inner synaptic layer of goldfish retina there are pharmacologically identifiable opioid pathways¹². The pathway to ON-centre ganglion cells seems to consist of at least an intrinsic peptidergic (opioid) neurone, which may release an enkephalin, and an intrinsic GABAergic amacrine cell which is inhibited by the opioid peptide and in turn inhibits the ganglion cell. The simplest scheme for such a pathway is shown in Fig. 3. Additional elements, such as excitatory interneurons between enkephalinergic amacrine (A_{ENK}) and GABAergic amacrine (A_{GABA}) or between A_{GABA} and the ON-centre ganglion cell, are possible. An overall effect of the presumed endogenous opioid transmitter, then, is to excite the ON-centre ganglion cells through disinhibition (opioid pathways to other ganglion cells may differ). This role of opioids in retinal ON pathways resembles their role elsewhere. For example, the immediate postsynaptic effect of enkephalins is generally inhibitory¹³, and the cells postsynaptic to enke-

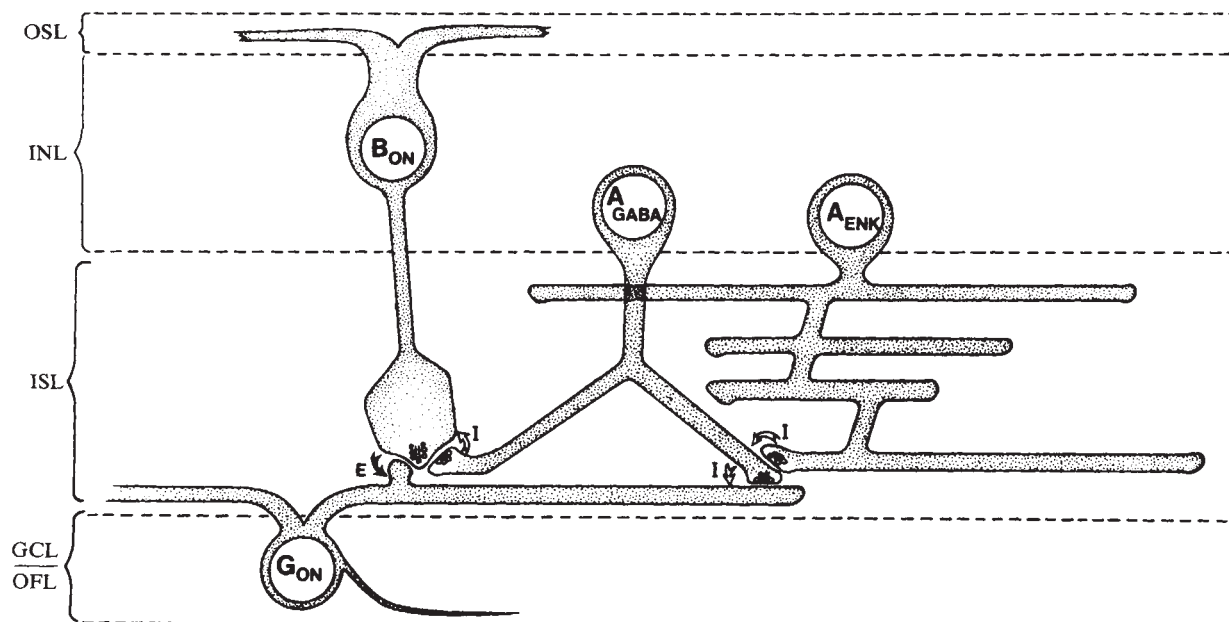


Fig. 3 The simplest probable hypothetical circuit of opioid and GABAergic neural pathways affecting ON-centre ganglion cells in the inner synaptic layer (ISL) of goldfish retina. Open arrows indicate inhibition (I); solid arrows indicate excitation (E). The presumed enkephalinergic amacrine (A_{ENK}) is inhibitory to the GABA amacrine (A_{GABA}), which in turn is inhibitory to the depolarizing bipolar (B_{ON}). The ON-centre ganglion cell (G_{ON}) may be inhibited by the GABA amacrine either directly or through excitatory input from the depolarizing bipolar. The nature of the synaptic input(s) to the presumed enkephalin cell are unknown. OSL, outer synaptic layer; INL, inner nuclear layer; GCL/OFL, ganglion cell/optic fibre layer.

phalinergergic neurones may themselves often be inhibitory¹⁴. In specific cases described recently, exogenous opiates inhibited the activity of GABA interneurons in the hippocampus¹⁴⁻¹⁶ and olfactory bulb¹⁶; the K⁺-evoked, Ca²⁺-dependent release of GABA from rat brain synaptosomes¹⁷, and the induced release *in vivo* of GABA from rat sensorimotor cortex¹⁸. These features are particularly clear in the goldfish retina which, because of its well-known neural organization and convenience as a natural 'brain slice', may be ideal for further testing of the organization of transmitter-specific neuronal assemblies such as these.

These results suggest interesting interpretations of the functional organization of the vertebrate retina. The precise function of amacrine cells is unknown, although they are generally supposed to have a critical role in controlling retinal output (ganglion cell responses)¹⁹. For example, in the inhibi-

tory interneurone, the GABAergic amacrine is connected and colour-coded in such a way as to impose a double colour-opponent surround upon the centred ganglion cell^{20,21}. The purpose of a second inhibitory interneurone, the presumed enkephalin amacrine, in modulating the effectiveness of this surround, is unclear. However, experiments on the role of enkephalin-releasing amacrine cells in the processing of visual information may elucidate the role of enkephalinergic neurones elsewhere in the central nervous system.

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Tonic *in vivo* inhibition of rabbit myometrial adrenergic receptors

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With the development of radioligand binding assays for identifying hormone receptors directly, it has become evident that the number of receptors in a particular cell or tissue does not remain constant but can in fact, be regulated by a variety of agents¹. Receptor concentrations decrease with exposure to homologous hormone (called desensitization or down-regulation) but may be affected differently by heterologous hormones. Thus in the adrenergic contractile response of rabbit myometrium, mediated by α -adrenergic receptors², the concentration of α -adrenergic receptors is regulated by sex steroids^{3,4}. This oestrogen treatment increases the concentration of myometrial α -adrenergic receptors approximately threefold over that measured in myometrium from immature rabbits, and, furthermore, is accompanied by an increased contractile response to catecholamines⁵. We report here that, after 24 h in organ culture, rabbit myometrium is more sensitive to α -adrenergic stimulation *in vitro*, and that particulate fractions prepared from cultured myometrium have a threefold increase in α -adrenergic receptors but not β -adrenergic receptors when compared with myometrium that has not been cultured. Our results suggest that the myometrium α -adrenergic receptor, in addition to being regulated by oestrogen, seems to be under tonic inhibitory control *in vivo* and that this control may only in part be accounted for by catecholamines.

Uteri from immature New Zealand rabbits (typically six rabbits per experiment) were pooled, the endometrium removed and myometrial strips divided equally into two groups. Myometrium in the first group was used immediately in isometric contraction studies or was homogenized and a particulate fraction prepared for binding studies, while myometrium in the second group was cultured. After 24 h in organ culture, strips were selected for isometric contraction studies or prepared for binding studies. ³H-dihydroergocryptine (DHE) was used to identify myometrial α -adrenergic receptors as described by Roberts *et al.*⁴ with several modifications (see Table 1). Binding of DHE to rabbit myometrium had been shown previously to meet the criteria for binding to the α -adrenergic receptor⁶. ³H-dihydroalprenolol (DHA) binding was found to be compatible with interaction with the β -adrenergic receptor.

Particulate fractions prepared from immature rabbit myometrium demonstrated a single class of binding site with low capacity and high affinity for DHE. After 24 h in organ culture, the concentration of DHE binding sites was significantly ($P < 0.005$) increased but the dissociation constant was unchanged (Table 1). The differences in the concentration of DHE binding sites in the particulate fractions prepared from cultured myometrium and myometrium that had not been cultured could not be attributed to either differences in numbers of cells as determined by DNA content⁷ of the homogenized strips (control, 5.06 ± 0.50 μ g DNA per mg wet weight; 24 h culture, 6.21 ± 0.67) or differential recoveries of protein⁸ (control, 1.6 ± 0.2 mg per g wet weight; 24 h culture, 1.4 ± 0.4) or plasma membrane as determined by 5'-nucleotidase activity (control, 2.83 ± 0.48 μ g P_i per mg protein per min; 24 h culture, 2.24 ± 0.41). 5'-Nucleotidase activity of the particulate fractions was determined by the method of Solymon and Trams⁹ at a protein concentration of 0.16 ± 0.20 mg ml⁻¹ for 20 min. Free inorganic phosphate was determined by the method of Chen *et al.*¹⁰.

DHE binding to the particulate fractions prepared from cultured myometrium retained the characteristics of α -adrenergic receptor interactions. The DHE binding site was stereoselective; the (-)-stereoisomer of adrenaline ($K_i = 0.2$ μ M) was 20 times more potent than the (+)-stereoisomer ($K_i = 4.0$ μ M) in competing for DHE binding. The order of

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Table 1 Dissociation constants and concentration of binding sites for DHE and DHA in rabbit myometrium

	DHE binding (n = 6)		DHA binding (n = 4)	
	Concentration of binding sites (fmol per mg protein)	Dissociation constant (nM)	Concentration of binding sites (fmol per mg protein)	Dissociation constant (nM)
Control	91 ± 10	2.6 ± 0.3	53 ± 6	0.5 ± 0.1
24 h organ culture	250 ± 6*	2.9 ± 0.6	46 ± 4	0.3 ± 0.1

Myometrium was cut into pieces not larger than 3×10 mm and washed in Ham's F-10 culture medium at room temperature. Myometrial strips (two to three per well) were cultured in 3 ml of serum-free Ham's F-10 culture medium with penicillin at 100 U ml^{-1} and streptomycin at $100 \text{ } \mu\text{g ml}^{-1}$ in a humidified 95% air/5% CO_2 atmosphere at 37°C . Particulate fractions were prepared as previously described⁴ except that the 10,000g centrifugation step was deleted from the protocol and dithiothreitol was omitted from the buffer used in the final pellet suspension. The DHE radioligand binding assay was used as previously described⁴ except that dithiothreitol was omitted from the assay tubes and instead ascorbic acid at a final concentration of 1.0 mM was used as an antioxidant, and the wash buffer used was 20 mM K_2HPO_4 , 1 mM MgSO_4 at pH 8.0. Final concentrations of DHE were 1–16 nM. The DHA radioligand binding assay was identical to the DHE assay except that the temperature of the wash buffer was 4°C and the wash volume was 25 ml. Final concentrations of DHA were 0.1–16 nM.

*Significantly ($P < 0.005$) different from control as determined by use of unpaired Student's *t*-test.

potency of agonist competition for binding to particulate fractions prepared from cultured myometrium—adrenaline ($K_i = 0.6 \pm 0.2 \text{ } \mu\text{M}$) $\geq$ noradrenaline ($K_i = 1.8 \pm 0.7 \text{ } \mu\text{M}$) $\gg$ isoprenaline ($K_i > 10 \text{ } \mu\text{M}$)—is identical to the order of potency for these agonists in stimulating contraction of rabbit myometrium². Inhibitory constants (K_i) were calculated from the relationship $K_i = \text{ED}_{50}/(1 + [\text{DHE}]/K_d)$, described by Cheng and Prusoff¹¹ and are presented in Table 1 as the $\bar{X} \pm \text{s.e.m.}$ from three experiments using particulate fractions from different rabbits.

The increased concentration of DHE binding sites in myometrium after 24 h in culture was accompanied by a significant increase in sensitivity to contraction induced by noradrenaline (Fig. 1a) and methoxamine, an α -adrenergic agonist subject to neither nerve re-uptake nor metabolism by catechol-*O*-methyltransferase (Fig. 1b). However, there was no change in sensitivity of cultured myometrium to acetylcholine (Fig. 1c).

The leftward shifts of the dose-response curves for noradrenaline- and methoxamine-induced contraction indicate that myometrial sensitivity to adrenergic stimulation increases during organ culture. The known examples of increased sensitivity or 'supersensitivity' have been divided into two major types¹². 'Deviation' supersensitivity, or prejunctional supersensitivity¹², is characterized by changes in the amount of agonist that is available to interact with receptors. Supersensitivity of this type would arise in our system if either decreased adrenergic neuronal uptake or decreased metabolism of amines

was present in the cultured myometrium. As cultured myometrium was also more sensitive to methoxamine-induced contraction it is unlikely that 'deviation' supersensitivity is the underlying mechanism.

'Nondeviation' supersensitivity, or postjunctional supersensitivity, as reviewed by Fleming *et al.*¹³, develops as a result of suppression of contact between neurotransmitter and its effector cell. Several different mechanisms have been proposed for postjunctional supersensitivity, including increased receptor concentration¹⁴. For example, in the rat depletion of neurotransmitter with reserpine leads to a leftward shift in the dose-response curve for noradrenaline- and carbachol-stimulated potassium release of submaxillary gland slices¹⁵ and this supersensitivity has been correlated with an increase in both α - and β -adrenergic receptor concentration¹⁶. In smooth muscle, postjunctional supersensitivity has been characterized by a nonspecific increase in sensitivity to various agonists acting on their specific receptors, and the response to changes in extracellular potassium and calcium ion concentration is altered^{14,17}. Our results show that cultured myometrium did not exhibit an increased sensitivity to acetylcholine, indicating that the observed increase in sensitivity is specific for α -adrenergically mediated contraction.

Table 2 Effect of the addition of (–)noradrenaline to the culture medium on the concentration of DHE binding sites

Treatment	DHE binding site concentration (% of control)
Control	100
8 h culture	145 ± 18
0.1 mM ascorbate	
8 h culture	101 ± 9
0.1 mM ascorbate	
1.0 μM (–)noradrenaline	

Myometrial strips were cultured as described in the text except that the culture medium was changed at 2-h intervals and after 8 h the strips were homogenized and particulate fractions prepared. In a preliminary experiment, Scatchard analysis indicated similar dissociation constants for DHE binding to particulate fractions prepared from myometrial strips cultured in the presence of either ascorbate or ascorbate and noradrenaline. Thus, it was unlikely that the particulate fractions prepared from noradrenaline-treated myometrium contained a higher concentration of noradrenaline which competed with DHE in the binding assay thereby producing an apparent decrease in DHE binding sites. To conserve tissue, the concentration of DHE binding sites was estimated by using a saturating concentration ($\sim 12 \text{ nM}$) of DHE and $10 \text{ } \mu\text{M}$ phentolamine to define nonspecific binding and was expressed as a percentage of DHE binding sites in myometrium from the same animals that had not been cultured ($n = 4$).

* Significantly different from strips cultured for 8 h with 0.1 mM ascorbate as determined by use of paired Student's *t*-test.

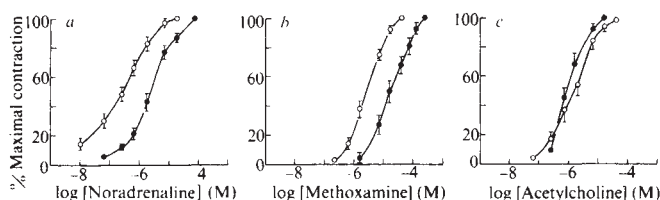


Fig. 1 Contractile response of myometrial strips that had not been cultured (●) and myometrial strips that had been cultured for 24 h (○) to cumulative doses of noradrenaline (a), methoxamine (b) and acetylcholine (c). Calculated ED_{50} ($\bar{X} \pm \text{s.e.m.}$) from individual experiments ($n = 10$) were as follows: a, ●, $2.8 \pm 0.6 \text{ } \mu\text{M}$; ○, $0.6 \pm 0.2 \text{ } \mu\text{M}$ ($P < 0.05$); b, ●, $22.2 \pm 4.0 \text{ } \mu\text{M}$; ○, $3.9 \pm 1.0 \text{ } \mu\text{M}$ ($P < 0.05$); c, ●, $3.8 \pm 1.7 \text{ } \mu\text{M}$; ○, $1.4 \pm 0.5 \text{ } \mu\text{M}$ (not significant). Statistical significance was determined by use of unpaired Student's *t*-test. Isometric contraction studies were carried out in isolated baths maintained at 37°C with Krebs–Ringer bicarbonate buffer (1.8 mM Ca^{2+} , glucose at 1 mg ml^{-1} , pH 7.4) and aerated with 95% $\text{O}_2/5\%$ CO_2 . Myometrial strips were suspended from a Grass FT03 strain-gauge transducer at an initial tension of 1 g for 1 h during which time the Krebs–Ringer solution was replaced at 15-min intervals. After 1 h, the tension was reduced to 0.25 g and the response recorded with a Grass physiograph. Response was calculated by integrating the area under the tracing for a standardized period of time.

Table 3 Effect of hexamethonium on myometrial DHE and DHA binding sites

Treatment	DHE binding sites (fmol per mg protein)	DHA binding sites (fmol per mg protein)
Control	80 ± 14	52 ± 13
10 mg per kg hexamethonium	159 ± 20*	72 ± 5†

Immature rabbits received five subcutaneous injections of hexamethonium (10 mg per kg) dissolved in 0.9% NaCl at 6-h intervals, were killed within 1 h of the last injection and their uteri removed. Preparation of myometrial particulate fractions and binding assays, including radioligand concentrations, were performed as described in Table 1 legend. Data are reported as the $\bar{X} \pm \text{s.e.m.}$

* Significantly ($P < 0.01$) different from control; unpaired Student's *t*-test.

† Significantly ($P < 0.05$) different from control; unpaired Student's *t*-test.

The observed increase of α -adrenergic receptors in cultured myometrium suggested that tonic inhibitory influences are present *in vivo*. The addition of noradrenaline (1.0 μM) to the culture medium prevented the increase in α -adrenergic receptors (Table 2). However, the results from this experiment alone do not indicate a regulatory role of catecholamines *in vivo*. To examine this possibility further, immature rabbits were treated with hexamethonium, a ganglionic blocking agent¹⁸, resulting in a significant increase in DHE binding sites ($P < 0.01$) and in DHA binding sites ($P < 0.05$).

The finding that noradrenaline prevented the *in vitro* increase in the concentration of myometrial α -adrenergic receptors whereas *in vivo* treatment of rabbits with hexamethonium resulted in an increase in the concentration of these receptors suggests that catecholamines may tonically down-regulate the α -adrenergic receptor *in vivo*. However, this may not be the only process involved, because the magnitude of the increase (99%) in α -adrenergic receptors with 24 h hexamethonium treatment was less than that (260%) observed during the organ culture over the same period of time. This difference could reflect an incomplete ganglionic blockade with the dose of hexamethonium used. However, there was no further increase in myometrial DHE binding sites (150 ± 14 fmol per mg protein; $n = 3$) of rabbits in which the dose of hexamethonium was increased to 20 mg per kg.

Thus, our studies suggest that in addition to regulation by sex steroids³⁻⁵, the myometrial α -adrenergic receptor of the immature rabbit seems to be under inhibitory control *in vivo* by endogenous catecholamines and an unidentified mechanism. The techniques described for organ culture of rabbit myometrium should facilitate investigation of the cellular mechanisms involved in the control of adrenergic receptors and their relationship to physiological response in this tissue.

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Monosynaptic muscarinic activation of K^+ conductance underlies the slow inhibitory postsynaptic potential in sympathetic ganglia

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Slow muscarinic inhibition, which lasts seconds or longer, is probably an important modulator of synaptic interactions in the central and peripheral nervous systems. Neither the cellular location of muscarinic receptors nor the ionic mechanism underlying the inhibition is well understood. In parasympathetic neurones of the cardiac ganglion in the mudpuppy, activation of muscarinic receptors leads to an inhibitory postsynaptic potential (i.p.s.p.) produced by an increase in membrane conductance to K^+ (ref. 1). At other sites, including sympathetic ganglia, however, the situation is less clear. In the 9th and 10th paravertebral sympathetic ganglia of the bullfrog stimulation of synaptic inputs to C neurones produces a slow muscarinic i.p.s.p.². From extracellular recordings, it was suggested³ that this slow i.p.s.p. is mediated through muscarinic excitation of an inhibitory, catecholamine-releasing interneurone. However, using similar methods, Weight *et al.*^{4,5} reported that muscarinic receptors are located on C neurones themselves. Further controversy exists over the ionic basis of the i.p.s.p.; stimulation of an electrogenic ion pump^{6,7} and reduction of membrane Na^+ conductance⁸ have both been proposed but remain unsubstantiated. We now present evidence, from intracellular recordings, that acetylcholine (ACh) produces a monosynaptic activation of muscarinic receptors located on sympathetic C neurones, and the i.p.s.p. is accompanied by an increase in membrane K^+ conductance.

Sympathetic chains, including ganglia 7-10, were isolated from 4-6-inch bullfrogs (*Rana catesbeiana*) and maintained *in vitro*. Spinal nerves 7 and 8, the sympathetic chain above ganglion 7 and the sciatic nerve were each fitted with suction electrodes to permit orthodromic and antidromic stimulation of ganglion cells. Cell bodies were impaled with microelectrodes made from 1.0-mm o.d. wall glass (Haer, WPI) and filled with 3 M KCl (resistances = 70-120 M Ω). A bridge circuit was used to estimate membrane potential while passing current through the microelectrode. Ionophoretic pipettes were filled with 1 M ACh (resistance 100 M Ω , backing current 1-4 nA). Micro-pipettes were positioned under visual control with Zeiss Nomarski optics ($\times 40$ water immersion objective). The preparation was superfused with Ringer's solution composed of 115 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM HEPES, pH 7.2. Dihydro- β -erythroidine HBr (DH β E, gift of Merck), *d*-tubocurarine Cl (*d*TC, Sigma) and atropine (Sigma) were applied by superfusion in the Ringer's.

Two classes of neurone are found in the 9th and 10th ganglia. They are identified by the origin and latency of the input producing a fast, nicotinic excitatory postsynaptic potential (e.p.s.p.). In agreement with others^{9,10}, we found that C neurones received their innervation from spinal nerves 7 and 8, whereas B neurones received theirs from higher spinal segments through the sympathetic chain. The latencies of nicotinic e.p.s.ps were 5-10 times greater in C neurones than in B neurones. In a given preparation, C neurones were generally much smaller than B neurones and cell size was used as a guide to impale C neurones preferentially. I.p.s.ps were never observed in a B neurone (see also ref. 2).

A single orthodromic shock, or a short train at 10 Hz, to an impaled C neurone in drug-free Ringer's elicited suprathreshold fast e.p.s.ps followed by a hyperpolarization of 5-25 mV, lasting 1-4 s (Fig. 1a). Superfusion with 100 nM atropine blocked the hyperpolarization which then recovered when atropine was

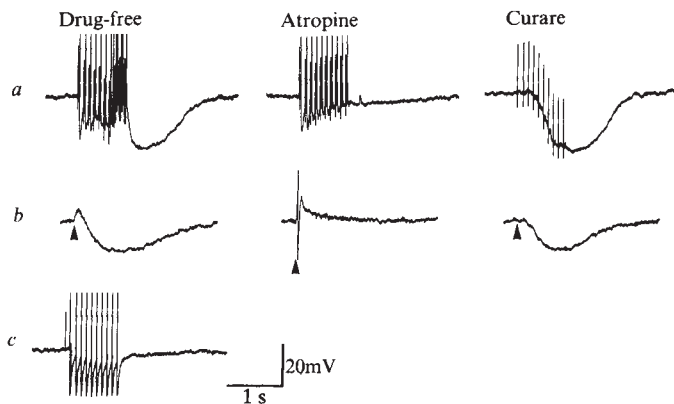


Fig. 1 Pharmacology of the i.p.s.p. *a*, Response of a C neurone to 10 stimuli at 10 Hz to nerves 7 and 8. *b*, After each synaptic trial a single iontophoretic pulse (15 nA, 5 ms) of ACh was applied (arrow). *c*, A train of antidromic action potentials elicited by 10 stimuli to the sciatic nerve. The tops of the action potentials in drug-free Ringer's and in 100 nM atropine have been cropped. Shock artefacts are evident in *a* and *c*. Atropine blockade was maximal by 10 min and was reversed by washing in drug-free Ringer's for 45 min. During atropine blockade, the nicotinic response to ACh became suprathreshold. After 20 min in 100 μ M dTC, the fast e.p.s.p. and ACh-evoked depolarization were blocked, revealing pure hyperpolarizing responses. Temperature = 27 $^{\circ}$ C.

washed out. The orthodromic hyperpolarization was distinctly larger than the summated afterpotential following a comparable train of antidromic action potentials (Fig. 1*c*). When the fast e.p.s.p. and consequent spike were blocked with either 4 μ M DH β E or 100 μ M dTC, a pure, atropine-sensitive, hyperpolarizing i.p.s.p. remained. These synaptic responses were mimicked by iontophoretically applied ACh (Fig. 1*b*). In normal Ringer's, a single pulse of ACh produced a biphasic response consisting of an early, depolarizing phase lasting 200 ms, followed by a larger, hyperpolarizing phase lasting 2 s. Atropine selectively abolished the hyperpolarization while curariform drugs blocked the depolarization. Thus the muscarinic i.p.s.p. is present in drug-free C neurones and can be mimicked by the application of ACh.

The possibility that an interneurone is involved in the generation of the i.p.s.p. was tested by measuring the effect of blocking synaptic release on the response to iontophoretically applied ACh in a curarized preparation (Fig. 2). The iontophoretic current was adjusted so that the ACh response closely mimicked the amplitude and time course of the i.p.s.p. Then the preparation was superfused with 0.18 mM Ca^{2+} , 8 mM Mg^{2+} Ringer's, which abolished the nerve-evoked slow i.p.s.p. but left the iontophoretic response unchanged. The synaptic response returned when normal Ringer's was reintroduced and both the i.p.s.p. and the ACh responses were then blocked completely with atropine. This demonstrates that the muscarinic action of ACh is independent of Ca^{2+} -sensitive release and is probably a direct effect on C cells.

The mechanism underlying the i.p.s.p. was examined by varying the membrane potential. In 20 cells, in which 10 stimuli at 10 Hz elicited an average i.p.s.p. of 12 ± 1.8 mV (s.e.m.) at resting potential -51 ± 1.5 mV (s.e.m.), polarizing current produced the effects illustrated in Fig. 3*a*. Depolarizing current reduced the amplitude and duration of the i.p.s.p. With progressive hyperpolarization, i.p.s.p. amplitude increased to a peak value, then became smaller and reversed. The i.p.s.p. reversed at -100 ± 4.2 mV (s.e.m.), $n = 4$ and the spike afterpotential reverses at -88 ± 2.4 mV (s.e.m.), $n = 5$ (for example Fig. 3*c, d*).

Interpretation of the voltage sensitivity of the i.p.s.p. requires information about the current-voltage (*I-V*) relationship of C

cells. Figure 3*b* is an *I-V* plot obtained by passing a slow current ramp through a balanced electrode. It is representative of data from five cells in which the average slope resistance increased sharply from 93 ± 14 M Ω (s.e.m.) to 380 ± 59 M Ω (s.e.m.) during hyperpolarization from rest. Over the linear, high-resistance range of the *I-V* curve, i.p.s.p. amplitude was a linear function of membrane potential and underwent reversal, suggesting a synaptically activated increase in membrane conductance. The curved portion of the *I-V* curve coincided with the range in which i.p.s.p. amplitude and spike afterpotential amplitude deviated from a linear dependence on membrane potential (compare Fig. 3*b* with *c* and *d*). In other words, both the i.p.s.p. and the afterpotential appeared to be shunted by a decrease in membrane input resistance at depolarized potentials.

The muscarinic conductance change was also investigated by passing 0.5-s current pulses across the membrane before and during the i.p.s.p. (not shown). As expected, there was always a decrease in membrane resistance during the i.p.s.p. over the linear, high-resistance part of the *I-V* relationship. In the region of transition from low- to high-input resistance we sometimes observed a resistance increase during the i.p.s.p. (see also refs 4, 8). However, this increase was consistently smaller than that produced by simply hyperpolarizing the membrane with injected current in the absence of an i.p.s.p. Thus despite the apparent decrease, the i.p.s.p. was always accompanied by an increase in membrane conductance. In three cells the conductance increase during the i.p.s.p. ranged from 1.6 to 3.9 nS. These estimates were made over the linear portion of the *I-V* plot and represent approximately a doubling of resting membrane conductance ($1/380$ M Ω = 2.6 nS).

The preceding experiments imply that K^{+} and/or Cl^{-} carry synaptic current. Such possibilities are usually distinguished by altering ion concentration gradients. The fact that use of KCl-filled microelectrodes had no discernible effect on the i.p.s.p. reversal potential suggests that Cl^{-} is not involved. In contrast, when extracellular K^{+} was increased from 2 to 10 mM, the reversal potential shifted to a substantially depolarized value (Fig. 4), indicating that K^{+} carries synaptic current.

Taken together, these experiments indicate a similarity between the muscarinic i.p.s.p.s of bullfrog sympathetic neurones and mudpuppy parasympathetic neurones: each involves a monosynaptic process dominated by an increase in K^{+} conductance. Furthermore, the inhibitory nature of this mechanism has been demonstrated. In some conditions (Fig. 3*a*, 0 pA and +40 pA) C neurones fired repetitively in response to a single antidromic shock and the i.p.s.p. blocked this firing. The

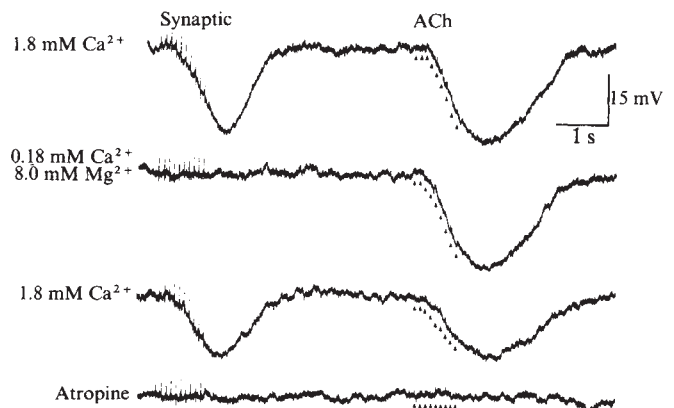
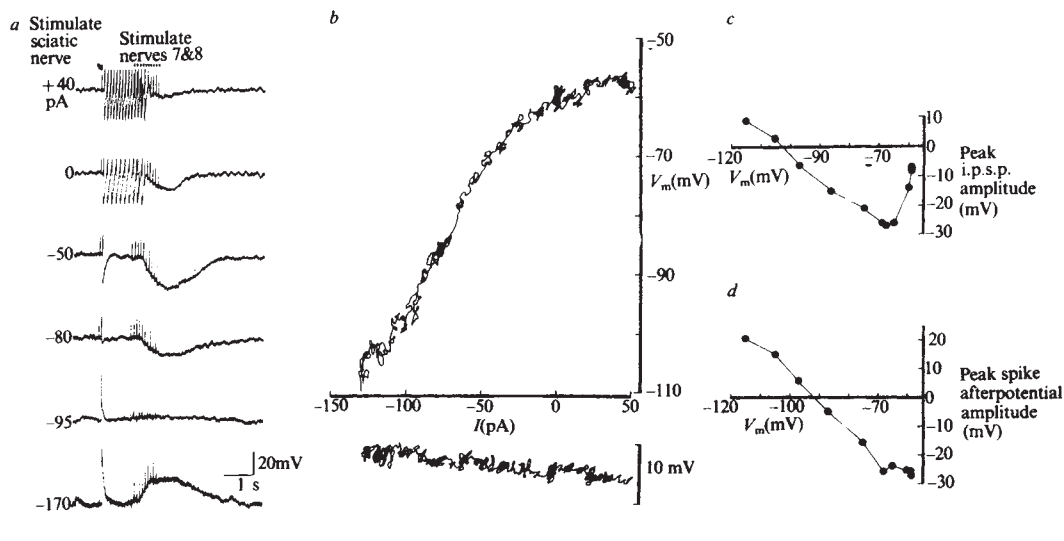


Fig. 2 Blocking synaptic release does not alter the muscarinic action of ACh on a C neurone. In normal Ringer's, containing 100 μ M dTC, the synaptic response (10 stimuli at 10 Hz to nerves 7 and 8) was mimicked by iontophoretically applied ACh (nine pulses of 20 nA, 4 ms, marked by arrows). After 10 min in 0.18 mM Ca^{2+} , 8 mM Mg^{2+} Ringer's, the synaptic response was selectively blocked. After 20 min of washing in normal Ringer's, the synaptic response returned. Both the synaptic and the ACh-evoked responses were completely blocked by 100 nM atropine. Temperature = 22 $^{\circ}$ C.

Fig. 3 Voltage sensitivity of the slow i.p.s.p. spike afterpotential and input resistance of a C neurone bathed in 100 μ M dTC. *a*, Intracellular recording of responses to a single antidromic shock followed by a short orthodromic train at different levels of polarizing current (left). At 0 pA and +40 pA the antidromic shock produced repetitive firing which was stopped by the i.p.s.p. (Control trials showed that the i.p.s.p. magnitude was unaffected by the repetitive firing.) *b*, Top, after balancing the bridge circuit of the microelectrode amplifier, a slow current ramp (such that $dV/dt < 4$ mV s^{-1}) was passed into the cell and used to construct an I - V curve by playing the data into an x - y plotter. Bottom, the electrode was then withdrawn from the cell and the same current ramp was passed through the electrode alone. Membrane potentials were estimated in the range over which the unbalanced electrode resistance was $< 10\%$ of the total measured intracellularly. The unbalanced electrode resistance was ~ 20 M Ω . Subtracting this from the top plot gives slope resistances of 135 M Ω at 0 pA and 505 M Ω at -100 pA. *c*, A graph of peak i.p.s.p. amplitude versus membrane potential (V_m). *d*, Graph of peak action afterpotential versus membrane potential. Note that the bend in the cell's I - V relationship coincides with the potential at which the relationships in *c* and *d* deviate from linearity. Temperature = 22 $^{\circ}$ C.



powerful influence of the i.p.s.p. on membrane properties suggests how monosynaptic inhibition might contribute to the integrative function of autonomic neurones.

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Recognition of H-2 domains by cytotoxic T lymphocytes

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The polymorphic major histocompatibility antigens (H-2) have a crucial role in the activation of antigen-specific T lymphocytes. Thus, H-2 antigens are not only recognized by allogeneic lymphocytes leading to generation of cytotoxic T lymphocytes (CTLs), but it has also been demonstrated that in syngeneic systems most T cells are only able to recognize foreign antigens in conjunction with their own MHC (major histocompatibility complex) antigens. This phenomenon, termed H-2 restriction¹, may be the key to our understanding of the biological function of MHC antigens. It is not clear whether recognition by T cells of H-2 on a molecular level is confined to particular domains on the H-2 molecule, nor whether the same polymorphic H-2 sites, which are characterized by antibodies, are recognized by allogeneic as well as by H-2-restricted syngeneic CTLs. Previous findings² indicate the existence of at least two major polymorphic domains on the H-2K^b molecule as defined by antibodies. Here we show the existence of CTLs with specificity for these polymorphic domains, and the preferential recognition of a particular domain by both alloreactive as well as H-2-restricted CTLs.

Recent competitive antibody-binding studies using six monoclonal antibodies against different determinants on the

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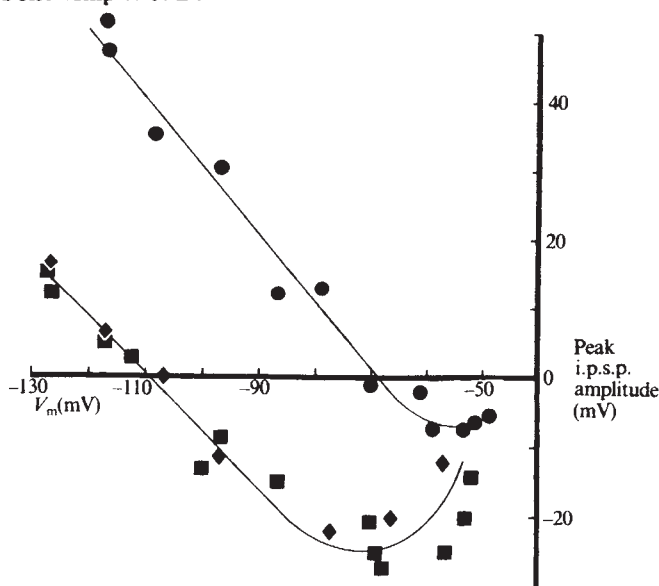


Fig. 4 K^+ dependence of the i.p.s.p. reversal potential. A plot of peak i.p.s.p. amplitude versus membrane potential. $\blacklozenge$, In 2 mM K^+ Ringer's, the i.p.s.p. reversed at -110 mV. $\bullet$, After 20 min. washing in 10 mM K^+ Ringer's, the i.p.s.p. reversed at -68 mV. $\blacksquare$, After 20 min washing in 2 mM K^+ Ringer's, the reversal returned to -110 mV. Lines were drawn by eye. Assuming intracellular K^+ concentration to be constant, the shift in K^+ equilibrium potential, calculated from the Nernst equation, would be 41 mV. Altering extracellular K^+ concentration produced no obvious change in either the resting potential (-51 mV) or the I - V relationship of this cell. At the i.p.s.p. reversal potential, the input resistance of the cell was 247 M Ω , after correcting for 24 M Ω of unbalanced electrode resistance. Temperature = 23 $^{\circ}$ C.

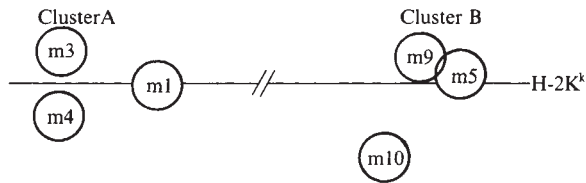


Fig. 1 Antigenic determinants on the H-2K^k molecule are concentrated in two clusters. This schematic diagram of the spatial relationship of H-2K^k determinants was obtained by antibody competition studies in which the binding of six different radiolabelled monoclonal antibodies to H-2K^k-bearing cells was competed by addition of various cold antibodies in all possible permutations. The 50% inhibition values have been used as a very rough measure of the relative distance between determinants on the H-2K^k molecule. Antibodies within one cluster cross-block each other to varying degrees but do not interfere with binding to the other cluster, as described in more detail elsewhere². Biochemical evidence indicates that all six determinants are located on the same molecule.

H-2K^k molecule³ have provided information on the steric relationship of individual determinants². The six H-2K^k determinants defined by monoclonal antibodies are concentrated on the H-2K^k molecule in two spatially separated polymorphic domains, clusters A and B, which are shown schematically in Fig. 1. We have used these monoclonal antibodies to block the target antigen for allogeneic as well as H-2-restricted CTLs to assess which H-2 sites are target epitopes for CTLs. H-2K^k-specific CTLs were produced *in vitro* by activation of DBA/2 splenocytes with irradiated A/J stimulator cells. Figure 2 shows the ability of different concentrations of monoclonal anti-H-2K^k antibodies to block target cell lysis. None of the monoclonal antibodies completely inhibited cytotoxicity, whereas a mixture of these plus a polyvalent anti-H-2K^k alloantisera almost completely blocked target cell lysis. These findings indicate that single monoclonal antibody bound to a particular determinant on the H-2K^k molecule prevents the interaction of only some, but not all, CTL clones with the target molecule, suggesting the existence of different CTL clones with specificity for distinct H-2 determinants. This conclusion has been supported by target inhibition of alloreactive as well as H-2-restricted CTL clones generated in a limiting dilution system which showed that individual monoclonal antibodies block only 10–50% of CTL clones (our unpublished data).

Figure 2 clearly shows that the monoclonal antibodies fall into two distinct groups which differ with regard to their inhibitory capacity. Antibodies belonging to cluster A (see Fig. 2a, centre panel) reduce ⁵¹Cr release from 80% to ~40% of the plateau level while those of cluster B reduce cytotoxicity to ~20%. The same difference is observed when the monoclonal antibodies are used at maximal inhibitory concentration (1:50 or 1:100 dilution), the ratio of killer cells to targets being varied (see Fig. 2b). Again monoclonal antibodies to cluster B are much more efficient in target inhibition, suggesting that more CTL clones are reactive against determinants belonging to cluster B than against those belonging to cluster A.

Next, we analysed an H-2-restricted CTL system in which CTLs from H-2^k mice recognize the hapten trinitrophenyl (TNP) linked to the H-2K^k molecule⁴. CBA splenocytes were sensitized *in vitro* with TNP-conjugated CBA spleen cells and cytotoxic activity was determined after 6 days of culture on TNP-conjugated H-2^k target cells. Control experiments established that the TNP-specific CTLs were predominantly restricted by the K end (data not shown). Inhibition of the TNP-specific CTLs by anti-K^k antibodies showed again that each individual antibody blocks only a fraction of the CTL effector population while a mixture of monoclonal antibodies or a polyvalent antiserum against K^k almost completely prevents lysis (see Fig. 3). These findings indicate that CTLs can recognize TNP in conjunction with different H-2 determinants, or in other

words, that one H-2K^k molecule carries more than one restriction site. As was also observed for allogeneic anti-K^k CTLs (Fig. 2), it was found for the TNP system that antibodies to cluster B were much more effective in preventing target cell lysis than antibodies to cluster A. The same conclusion was reached when the antibody concentration was constant at maximal inhibitory concentration (1:50–1:100 dilution) and the killer cells titrated (data not shown).

Calculation of lytic units provides an estimate of the relative number of CTLs in a population⁵. These data (see Table 1) show that in the presence of monoclonal antibodies to cluster B, on average three times fewer lytic units are measured than in the presence of monoclonal antibodies to cluster A. Taken together, these data suggest that about three times as many alloreactive and self-restricted CTLs are present which react with determinants of cluster B.

Target inhibition by monoclonal antibodies has also been described by others^{6,7} but our report provides additional information on the correlation of CTL specificity with the steric arrangement of H-2 determinants. The data show that both alloreactive as well as TNP-specific H-2-restricted populations are heterogeneous with regard to which H-2 epitopes on a given molecule are recognized, and that both CTL populations show preference for a particular domain on the H-2K^k molecule which is characterized by antibodies to cluster B. It is not clear from our results whether CTLs recognize exactly the same determinants as monoclonal antibodies or whether the latter block spatially related determinants only by steric hindrance. Another possibility is that monoclonal antibodies induce con-

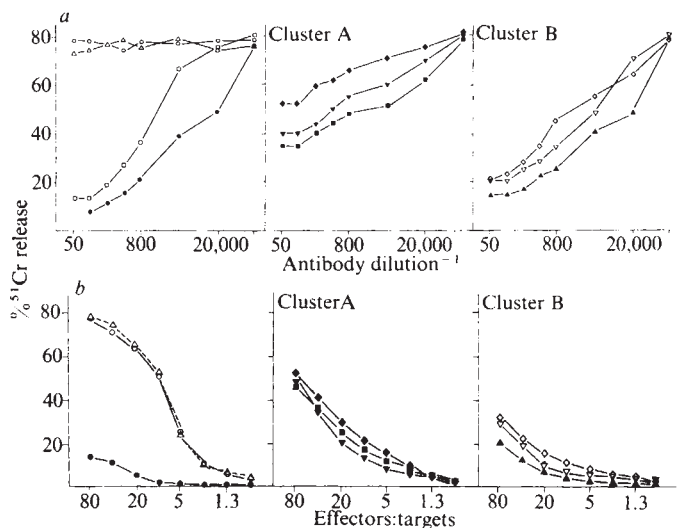


Fig. 2 Target inhibition of H-2K^k-specific alloreactive CTLs by monoclonal anti-H-2K^k antibodies. 10×10^6 DBA/2 (d/d) spleen cells were stimulated with 12×10^6 A/J (k/d) irradiated spleen cells as described elsewhere⁹. After 5 days, CTL activity was measured against ⁵¹Cr-labelled L929 (H-2^k) target cells for 4 h in the presence of anti-K^k antibodies in a total volume of 200 μ l using methods described previously⁶. These CTLs were shown to be specific for K^k (results not shown). In some experiments B10.D2 responders and B10.A stimulators were used. The figure shows a typical experiment which was repeated several times with the same results. **a**, Effect of different concentrations of monoclonal antibodies ascites on cytotoxicity at an effector to target ratio of 50:1. Left frame: $\circ$, medium control; Δ , control ascites 13/18; $\square$, hyperimmune BALB/c anti-A/J alloantisera; $\bullet$, mixture of six monoclonal anti-K^k antibodies. Middle frame, anti-K^k antibody describing cluster A: ∇ , m3 = H100-5R28; $\blacksquare$, m4 = H100-27R55; $\blacklozenge$, m1 = H116-22R7. Right frame, monoclonal anti-K^k antibody describing cluster B: $\diamond$, m5 = H100-30R23; ∇ , m9 = H142-23/3; $\blacktriangle$, m10 = H142-45/2. **b**, CTL titration in the presence of a constant amount of blocking antibodies (1:50 dilution of ascites). Same symbols as for **a**.

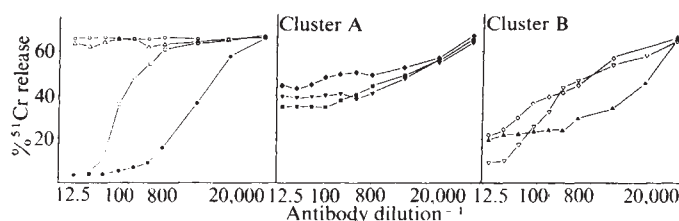


Fig. 3 Target inhibition of TNP-specific H-2K^k-restricted CTLs from CBA (H-2^k) mice by monoclonal anti-K^k antibody. TNP-specific CTLs were produced *in vitro* by activation of CBA splenocytes against TNP-conjugated CBA spleen as described elsewhere⁴. CTL activity was measured at an effector to target ratio of 50:1 against TNP-conjugated ⁵¹Cr-labelled L929 (H-2^k) target cells in the presence of various concentrations of blocking antibodies. By using different targets as well as cold target inhibition it was established that most CTLs were restricted by K^k. From other studies it is known that the antibodies used here mediate their blocking effect by inhibition of the target determinant and not when they are directed against the effector cells⁶. Symbols are the same as for Fig. 2. Left frame: ○, medium control; △, control ascites 13/18; □, BALB/c anti-A/J alloantiserum; ●, mixture of six monoclonal anti-K^k antibodies. Middle frame, ascites of monoclonal antibody to cluster A (m1, m3, m4); right frame, monoclonal antibody to cluster B (m5, m9, m10).

formational changes at distal parts of the H-2 molecule which influence the interaction with CTLs. However, we consider it more likely that both CTLs and antibodies recognize basically the same polymorphic domains. The different blocking capacity of monoclonal antibodies to clusters A and B is not likely to be due to distinct physical properties of the monoclonal antibodies because those used here have similar affinities (ref. 8 and H. Lemke, personal communication) and belong to similar immunoglobulin classes (IgG2a and IgG2b, respectively). An exception is H145-45/2, which after recent re-evaluation was found to be IgM (unpublished data); the fact that it is pentameric may explain its very strong inhibitory capacity.

A possible explanation for the striking preference of alloreactive and H-2-restricted CTLs for cluster B is that both H-2^d (DBA/2 or B10.D2) and H-2^k mice use a similar or related pool of T-cell receptors for recognition of the K^k molecule, whether an alloantigen (in the case of DBA/2) or a self antigen (in the case of CBA) is recognized. Such a hypothesis would be compatible with dual recognition models, one binding site being for TNP and the other for H-2 determinants. Arguments in favour of single recognition models assume that neodeterminants formed by TNP modification of either H-2 or other surface components are predominantly located in the

direct vicinity of polymorphic H-2 domains. Target inhibition of restricted CTLs by anti-H-2 would then be caused by steric hindrance. Note that preference of different CTL populations for the same cluster could be merely coincidental as it is possible that an H-2 molecule carries only few regions which, for steric reasons, are accessible to T cells. Further analysis of K^k-restricted CTL systems will clarify this question.

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Light chain isotypes selectively associate with heavy chain idiotypes in T-dependent and T-independent dextran-specific precursors

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In murine immunoglobulins, the κ chain is the major light chain isotype, as exemplified by the small amount of λ immunoglobulins present in normal sera, the rare occurrence of λ plasmacytomas and the preponderance of κ light chains in induced antibodies. Notable exceptions to this are the response to $\alpha(1 \rightarrow 3)$ dextran¹ in BALB/c mice and the heterocytic antibodies raised against the nitrophenol hapten in C57BL/6 mice². In the former response, the expression of idiotype in the hypervariable regions of the heavy chain³ is characteristically restricted. We have now analysed the B-cell precursors for the $\alpha(1 \rightarrow 3)$ linkage-specific response induced by T-dependent and T-independent dextran antigens with respect to light chain isotype and heavy chain idiomorph expression. Our findings clearly demonstrate that the two forms of dextran antigen trigger different B-cell precursor subpopulations. Furthermore, each individual animal appears to possess a different repertoire of idiomorph-committed precursors. This contrasts with the response of BALB/c mice to phosphorylcholine (PC) where most anti-PC antibodies and precursors respond with the dominant expression of the T15 idiomorph family, regardless of whether T-dependent or T-independent PC antigens are used⁴.

BALB/c mice immunized with the T-independent antigen dextran B1355s respond with the expression of unique idiotype (IdI) J558 and M104E, and a cross-reacting IdX idiomorph⁵. Schilling and co-workers⁶ have shown that the unique IdI specificities are generated during the V-D-J joining process of heavy chain genes. While the expression of λ chains in the T-independent anti-dextran BALB/c response is consistently >90%, the amount of J558 and M104E IdI idiotype varies between individual animals^{7,8}. This finding led us to speculate that the variable expression of heavy chain idiotype might be caused by individually different, idiomorph-specific regulation of B-cell precursors. Alternatively, during the development of the dextran-specific repertoire individually different exposures to environmental antigens could influence precursor maturation. According to the latter hypothesis, serum expression of light

Table 1 Lytic units of alloreactive and self-restricted CTL effector cells in the presence of blocking antibody against target determinants

Anti-H-2 antibody added to effector phase	H-2 cluster recognized	Source of CTLs	
		DBA $\bar{a}$ A/J	CBA $\bar{a}$ CBA-TNP
No antibody	—	10.0	14.1
13/18 (1a.7)	—	10.0	13.2
H100-5R28 (m3)	A	1.03	2.6
H100-27R55 (m4)	A	0.84	1.9
H116-22R7 (m1)	A	1.33	4.35
H142-23/3 (m9)	B	0.35	0.54
H100-30R23 (m5)	B	0.35	0.82
H142-45/2 (m10)	B	0.12	0.72
Mixed monoclonal antibodies	A + B	0.02	<0.01

Lytic units (LU) were calculated according to Cerottini *et al.*⁵ on the basis of CTL effector titrations as shown in Fig. 2b for alloreactive CTLs. The corresponding titration curves for TNP-specific CTLs are not shown. One lytic unit is defined as the number of effector cells required to lyse 50% of the target cells.

Table 1 Co-expression of idiotypes and light chain isotypes by individual dextran-specific precursors

Group	Co-expression* of	By T-independent clones† (%)	Expected precursor doublets‡	By T-dependent clones (%)	Expected precursor doublets
1	λ and IdX	73 (41/56)	—	24 (12/51)	—
2	λ and J558 IdI	11 (14/129)	—	5 (9/182)	—
3	λ and M104E IdI	7 (9/129)	—	2 (3/182)	—
4	κ and IdX	Not found (0/56)	—	Not found (0/51)	—
5	κ and J558 IdI	Not found (0/56)	—	Not found (0/51)	—
6	κ and M104E IdI	Not found (0/56)	—	Not found (0/51)	—
7	λ , J558 IdI and IdX	5 (3/56)	0.5/56	6 (3/51)	0.1/51
8	λ , M104E IdI and IdX	Not found (0/56)	0.3/56	Not found (0/51)	0.03/51
9	λ , M104E and J558 IdI	0.77 (1/129)	0.1/129	Not found (0/182)	0.02/182

* Dextran-specific precursors were analysed for the co-expression of the given light chain isotype and the heavy chain idiotype.

† Splenic fragments were immunized with either dextran B1355s (T-independent-2) or dextran-haemocyanin (T-dependent). Supernatants were collected every 3–4 days and analysed for total anti-dextran antibodies. Positive wells were selected and analysed for the co-expression of the appropriate light chain isotype and heavy chain idiotype. The numbers in parentheses represent the number of clones which co-express the isotype and idiotype out of the total number of positives examined.

‡ The statistical probability that the two given idiotypes will be co-expressed, based on the relative frequency of each idiotype alone.

chain isotypes and heavy chain idiotypes should directly reflect the differently established primary B-cell repertoire in individual animals.

Spleen cells from normal BALB/c mice were transferred in limiting cell numbers to 1,400 rad-irradiated normal or haemocyanin-primed syngeneic recipients. Twenty hours later, splenic fragment cultures were prepared according to the technique of Klinman⁹, modified for the analysis of T-dependent and T-independent B-cell precursors⁴. Fragment cultures were immunized with either dextran B1355s or dextran coupled to haemocyanin as described previously¹⁰. Culture supernatants were collected at 3-day intervals beginning on day 6 of culture and assayed for light chain isotype, heavy chain idiotypes and total anti-dextran antibody production, using enzyme-linked assays or radioimmunoassays (RIPs). Monoclonal antibodies were used to detect the cross-reacting IdX idiotype and the J558 and M104E IdI idiotypes¹¹. Figure 1 shows the percentage of heavy chain idiotypes and light chain isotypes calculated from data on 182 T-dependent and 129 T-independent dextran-

specific precursors. Precursor clones responding to the $\alpha(1 \rightarrow 6)$ linkage, present in dextran B1355s, were excluded by testing for inhibition with dextran B512 [$\alpha(1 \rightarrow 6)$] in the anti-dextran RIA¹⁰. The data show that the IdI-producing clones in the T-dependent and T-independent anti-dextran precursors are infrequent; most T-independent precursors express the cross-reacting IdX heavy chain idiotype while T-dependent precursors express the IdX idiotype in <25% of dextran-specific clones. Furthermore, T-dependent precursors express the κ chain more frequently than do T-independent precursors. The ability of T-dependent precursors to express both λ and κ light chains could explain why the frequency of dextran precursors responding to the T-dependent antigen is higher than that of T-independent precursors¹⁰. Although the relative fraction of IdX precursors is smaller in the T-dependent than in the T-independent response, the absolute number of IdX precursors does not appear to differ much for T-dependent and T-independent dextran antigens. Thus the selection of heavy and light chains is dictated by the type of dextran antigen.

The question of whether heavy chain idiotypes can freely combine with λ and κ light chains or whether their association is restricted cannot be answered from the collective precursor analysis data shown in Fig. 1. Therefore, individual clones were grouped together according to their co-expression of light chain isotypes with heavy chain idiotypes. The relative frequencies of clones co-expressing a certain light chain with a certain idiotype were calculated from the total number of T-independent and T-dependent dextran-specific clones. The data are shown in Table 1. The most frequent combination is the co-expression of λ and IdX in T-independent and T-dependent precursors (group 1). No precursors were found which co-expressed κ and any of the heavy chain idiotypes. A small number of T-independent and T-dependent precursors were positive for J558 IdI and IdX; none was positive for M104E IdI and IdX. These data demonstrate that the J558-M104E IdI idiotype site is different from the IdX site, in agreement with the assignment of IdI and IdX sites in hybridoma anti-dextran antibodies made by Schilling *et al.*⁶. The failure to find M104E IdI- and IdX-positive clones is not surprising considering the rare occurrence of M104E IdI precursors. However, the failure to detect known heavy chain idiotypes, that is, M104E, J558 and IdX, in precursors expressing the κ light chain is striking.

It is important for the evaluation of fragment cultures secreting more than one idiotype antibody to determine the probability of two different clones being present in one fragment. The expected frequency of clonal precursor doublets was calculated from the number of single idiotype-secreting cultures. The finding of a clone expressing J558 and M104E is unexpected, and unlikely to result from the presence of two different clones present in a single fragment culture as the likelihood of a clonal doublet at the given experimental limiting cell dose is >1 in 1,000 (see group 9 in Table 1; 0.1 in 129 T-independent clones).

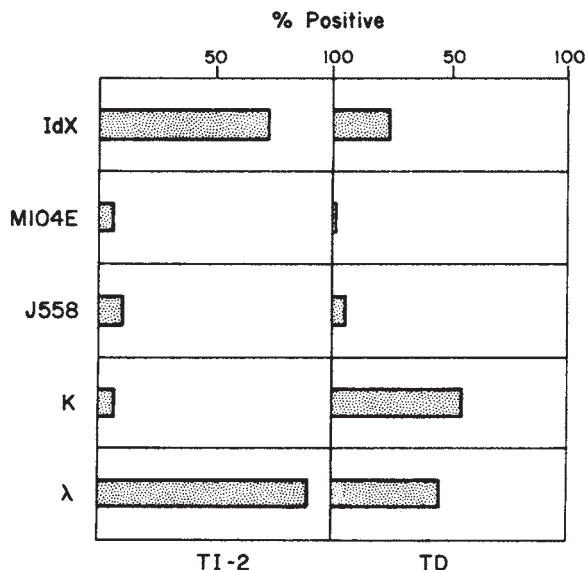


Fig. 1 The per cent positive dextran-specific precursors is plotted against the heavy chain isotype and the light chain idiotypes. Splenic fragments were immunized with either dextran B1355s (T-independent (TI)-2) or dextran-haemocyanin (T-dependent, TD). Supernatants were collected every 3–4 days and analysed for total anti-dextran antibodies, light chain isotype and heavy chain idiotype by RIA and enzyme-linked immunosorbent assay using monoclonal anti-J558 EB3-7-2, $\gamma 1\kappa$, raised in A/J mice, monoclonal anti-M104E SJL 18-1, $\mu\kappa$, raised in SJL mice, and monoclonal anti-IdX CD3-2, $\gamma 1\lambda$, raised in A/J mice.

Table 2 Analysis of dextran-specific precursors in individual BALB/c mice

Animal no.	Precursor per 10 ⁵ B cells		% J558 IdI		% M104E IdI		% IdX	
	TI	TD	TI	TD	TI	TD	TI	TD
1	4.50	4.36	<4(0/25)	23(3/13)	20(5/25)	<8(0/13)	ND	ND
2	2.00	7.41	25(3/12)	5(1/21)	<8(0/12)	<5(0/21)	ND	ND
3	0.80	1.60	<20(0/5)	<20(0/5)	<20(0/5)	<20(0/5)	ND	ND
4	0.80	5.49	<20(1/5)	<6(0/16)	20(1/5)	<6(0/16)	ND	ND
5	1.82	6.99	10(1/10)	<5(0/19)	20(2/10)	<5(0/19)	ND	ND
6	1.39	5.66	<6(0/17)	<3(0/32)	<6(0/17)	6(2/32)	86(12/14)	19(3/16)
7	1.48	3.65	<4(0/25)	5(1/22)	<4(0/25)	5(1/22)	95(20/21)	24(5/21)
8	3.51	ND	38(3/8)	ND	<13(0/8)	ND	38(3/8)	ND
9	ND	6.82	ND	4(1/22)	ND	<4(0/22)	ND	16(4/25)
10	0.32	7.81	50(1/2)	9(3/22)	<50(0/2)	<5(0/22)	ND	ND
11	0.97	1.60	<17(0/6)	<20(0/5)	<17(0/6)	<20(0/5)	ND	ND

Normal or haemocyanin-primed BALB/c mice were irradiated with 1,400 rad and used as recipients of individual adult syngeneic cells. Splenic fragments were immunized with dextran B1355s or dextran-haemocyanin and supernatants were collected every 3–4 days and analysed for total anti-dextran antibodies. Dextran-specific precursors were analysed for J558 IdI, M104E IdI and IdX idiotype as described in Fig. 1 legend.

It would be attractive to assume for the double IdI-positive clone that both chromosomes have made a productive but different DNA rearrangement resulting in two active genes producing J558 and M104E messages.

We have previously^{7,8} observed variation of the amount of heavy chain IdI idiotypes in individual mice immunized with dextran B1355s. For example, the amount of J558 IdI in four randomly selected BALB/c mice 7 days after immunization with dextran B1355s varied from 7 to 53% of the total anti-dextran response. To determine whether this individually different idiomorph expression is the result of idiomorph-specific regulation, induced by antigenic stimulation, or is predetermined by the number of idiomorph-committed precursors, precursor analysis of individual BALB/c mice was performed. Table 2 shows that individual mice indeed express the IdI idiotypes in significantly different amounts for T-dependent and T-independent responses. The relative J558 IdI frequency for T-independent precursors in individual animals was 4–50% and for T-dependent J558 IdI 3–23% (see Table 2). The overall (averaged) expression of J558- and M104E-positive precursors, as well as the number of T-dependent and T-independent responding precursors, is in good agreement with the data from analysis on precursors from pooled cell donors.

The present findings lead to three conclusions important for the understanding of mechanisms controlling idiomorph expression. (1) Certain heavy chain idiotypes seem to depend on the combination with one kind of light chain. (2) Different antigenic forms of the same antigenic determinant can trigger different precursors: T-dependent dextran antigen triggers significantly more κ precursors than the T-independent dextran B1355s. Conceivably, T cells, which participate in the T-dependent response, help to recruit κ chain B-cell precursors. (3) Individual mice possess variable frequencies of precursors expressing different idiomorphic specificities. Although the individually variable expression of idiotypes after immunization^{7,8} has not been formally linked to variable idiomorph precursor frequencies, we could infer that individual idiomorph expression stems directly from the presence of variable precursor compositions. Accordingly, individual variation of precursor frequencies must be generated during the maturation of the B-cell repertoire in the fetal-neonatal period¹². Idiomorph-specific factors might be important in controlling the development of B-cell repertoires¹³. In addition, isotypic network interactions control the expression of idiotypes in the adult animal during primary or established immune responses^{14–17}. Comparison of the immature with the mature idiomorph repertoire will help to define the role of regulatory mechanisms in establishing clonal immune profiles¹⁸.

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Independent control of immunoglobulin heavy and light chain expression in a murine pre-B-cell line

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B lymphocytes differentiate from multipotential stem cells by an unknown number of intermediate stages. Although the earliest stages of this sequence remain obscure, cells, which appear to be intermediate in this process, have been described^{1–7}. Large, proliferating, rapidly sedimenting cells which synthesize immunoglobulin chains that can be easily detected in the cytoplasm (cIg) but are either absent or less stable on the cell surface (sIg) have been found in both fetal liver and adult bone marrow. Smaller cIg⁺ sIg[−] cells have also been described^{2,5,7}. These cells are designated pre-B cells and may comprise a functionally heterogeneous group of progenitors⁸. Several cell lines, which share many properties with these postulated precursors, have been useful in establishing an asynchronous onset of immunoglobulin expression characterized by the appearance of μ heavy chains in the absence of light chain expression^{10–12}. We confirm here that the mitogen-responsive pre-B-like cell line, 70Z/3, in normal growth conditions, only expresses heavy chain, although it has a fully rearranged light chain gene¹³. 70Z/3 cells can also display the μ chain on the cell surface in the absence of light chain induction, most notably after exposure to dextran sulphate.

The 70Z/3 cell line synthesizes immunoglobulin detectable only intracellularly, but on exposure to lipopolysaccharide (LPS) the cIg⁺ sIg⁻ cell type rapidly acquires sIg⁺. Concomitant with the induction of sIg is the appearance of κ light chains¹⁴. There is, however, no evidence for secretion⁹. The increase in κ chain synthesis is related to induction of κ mRNA¹⁵.

The initial hypothesis, that light chain synthesis is causally related to surface acquisition of immunoglobulin, is not necessarily the case. This was demonstrated using a second B-cell mitogen, dextran sulphate (DxS). As shown in Fig. 1, exposure to LPS led to the acquisition of both μ and κ , whereas exposure to DxS resulted in a similar level of surface μ expression without expression of κ chains. Furthermore, the kinetics of μ expression differ after exposure to these two mitogens. Figure 2 shows that the detection of μ after DxS stimulation preceded that of both the μ and κ induced after LPS stimulation although ultimately similar levels were achieved. DxS does not, however, inhibit κ induction by LPS (data not shown).

Exposure to these mitogens neither altered the rate of proliferation, nor caused cell death and, although the kinetics of induction were variable, in most experiments >90% of the cells were sIg⁺ within 24 h (two cell cycles). It thus seems unlikely that different subpopulations of cells account for these results. Furthermore, most clones of 70Z/3 cells also respond to both DxS and LPS (see below), and the relative sensitivity of the reagents used to detect μ and κ chains was found to be similar.

We observed that whereas the μ and κ found on the membrane of LPS-stimulated cells was distributed over most of the cell surface, the μ chains detected on DxS-stimulated cells were usually found in tight caps. These tight caps were partially inhibited (95% capped reduced to 50%) by prior treatment with

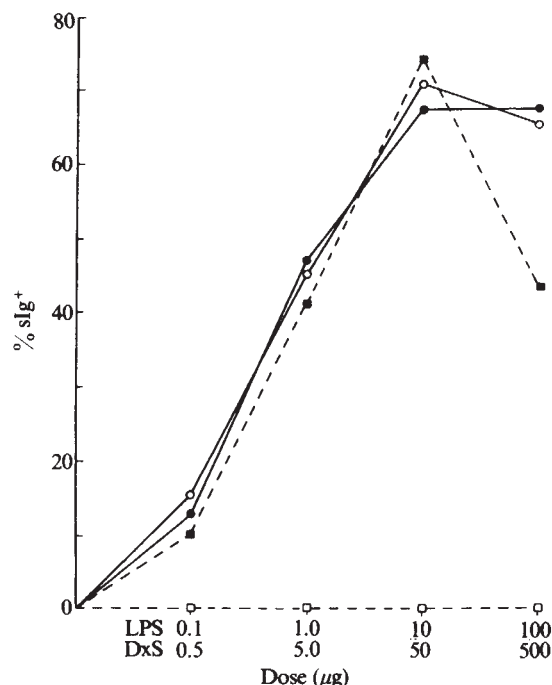


Fig. 1 Increase in surface immunoglobulin expression in 70Z/3 cells exposed to various concentrations of LPS (*Salmonella typhosa* WO 901, Difco) or DxS (Pharmacia) for 36 h. Surface expression of either μ or κ chains was assessed using affinity-purified, rhodamine-conjugated goat antibodies specific for either μ or κ which were prepared and used as previously described⁹. ●, LPS, μ ; ○, LPS, κ ; ■, DxS, μ ; □, DxS, κ ; 70Z/3 cells were grown in RPMI-1640 containing 10% FCS (Associated Biomedic Systems) and supplemented with 5 mM glutamate, 10^5 U⁻¹ penicillin, 0.1 g⁻¹ streptomycin and 5×10^{-5} M 2-mercaptoethanol. Cell cultures were initiated at 2.5×10^5 ml⁻¹ grown in 25-cm² tissue culture flasks (Corning) and kept at 37 °C in a humid atmosphere containing 7% CO₂.

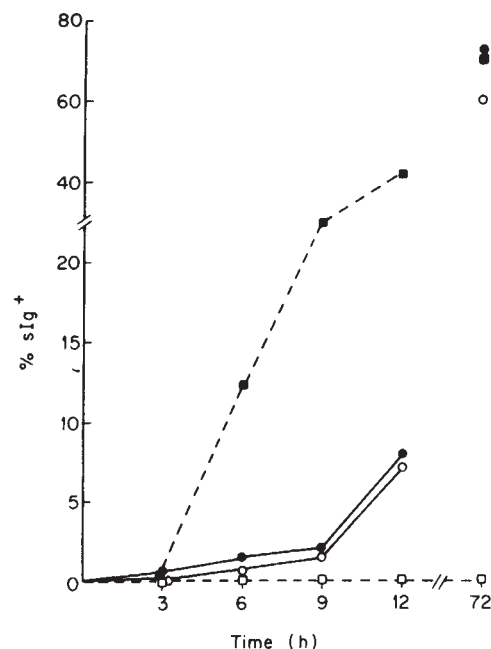


Fig. 2 Difference in time of appearance of cell-surface immunoglobulin chains after stimulation with LPS ($10 \mu\text{g ml}^{-1}$) or DxS ($50 \mu\text{g ml}^{-1}$). ●, LPS, μ ($10 \mu\text{g ml}^{-1}$); ○, LPS, κ ($10 \mu\text{g ml}^{-1}$); ■, DxS, μ ($50 \mu\text{g ml}^{-1}$); □, DxS, κ ($50 \mu\text{g ml}^{-1}$). Cell cultures were initiated at 2.5×10^5 ml⁻¹ in 25-cm² tissue culture flasks.

cytochalasin B (30 min, 37 °C, $10 \mu\text{g ml}^{-1}$) but not by staining in the presence of 1% NaN₃.

Possible explanations for the different cell surface distribution of μ chains after exposure to LPS or DxS include that: (1) the μ appearing after DxS stimulation differs from the μ found after LPS stimulation; (2) the presence of κ chains, although not necessary for the appearance of μ on the surface, is required for the stability of the sIg; or (3) a cell-surface alteration unrelated to immunoglobulin molecules may be responsible. It is also possible that the uninduced cells possess a small but undetectable amount of surface μ which undergoes a conformational change on exposure to DxS, leading to enhanced expression. Biochemical evaluation of the DxS- and LPS- induced μ is underway and may resolve these issues.

Additional differences in the responses to these two mitogens was found by analysing clones of 70Z/3 obtained by growth in soft agar. Although 19 of these clones (70Z/3.2–70Z/3.20) had similar characteristics to the parent line and could respond to both LPS and DxS, one clone, 70Z/3.1, lost the ability to respond to LPS, although retaining DxS responsiveness (Table 1). This clone was refractory to even 100 times the concentration of LPS normally used. Furthermore, the parent line and LPS-responsive clones, if grown in the presence of serum-free medium, failed to respond to LPS although the DxS response was normal (Table 1). After an initial period of adaptation, growth in this medium was similar to growth in serum-containing medium and LPS responsiveness was restored by addition of 2% fetal calf serum (FCS). This may indicate that serum contains components which condition the cells for responsiveness to LPS. Previously we found that different batches of either FCS or newborn serum themselves could cause background levels of sIg⁺ cells to vary from 1 to 99% (ref. 9, and unpublished data). It should be noted that in almost all these cases of elevated background expression of μ , light chains were not detectable. Thus, DxS is not obligatory for the detection of the 'μ only' surface phenotype. Furthermore, recent data suggest that the surface μ is indeed membrane μ and not secretory. First, similar to our previous report with LPS-induced cells, the supernatants of 70Z/3 cells exposed to DxS contained no detectable immunoglobulin (C. Sidman and C. J. P., in preparation). Second, biochemical analysis of the μ proteins of

70Z/3 cells reveal (1) a cell-surface form, labelled by lactoperoxidase-catalysed iodination, which corresponds in size to membrane μ characteristic of small resting B cells, and (2) biosynthetically labelled intracellular forms which correspond to the smaller precursors of both the secretory and membrane pathways (ref 16, and C. Sidman and C. J. P., in preparation). Analysis using tunicamycin of the non-glycosylated products further revealed a precursor pattern previously described for both membrane and secretory μ ¹⁶. In this respect 70Z/3 cells are similar to other B cells containing the precursors for both pathways but the final product of only the membrane form¹⁶.

The detection of $\text{clg}^+ \text{slg}^-$ cells which acquire sIg confirms the circumstantial evidence which suggests that this transition normally occurs during B-cell differentiation^{7,17,18}. Our findings are also consistent with reports that the onset of heavy and light chain synthesis is asynchronous^{12,19}. It is not clear whether the expression of μ chains has a regulatory role in B-cell development before light chain appearance. One report suggests that some pre-B cells may be capable of secreting μ chains¹⁹. Our findings, although different in that 70Z/3 cells do not secrete their μ chains, also suggests the possibility of interaction with the external environment through the variable region of the μ chain. This interaction might occur before synthesis of complete antibody molecules and thus be relevant to theories of clonal abortion, tolerance induction and network formation. Maki *et al.* have recently shown that whereas μ only fetal liver hybridomas have unrearranged light chain genes, the κ gene of ' μ only' 70Z/3 has already undergone rearrangement¹³. Thus several stages of pre-B cells may exist based on both DNA molecular arrangement and the expression of rearranged gene products.

We do not yet know the incidence of precursors in fetal and adult tissues with properties corresponding to 70Z/3 cells. These cells may represent a transient stage of B-cell maturation which is subject to regulation by factors such as those present in certain serum lots and by mitogens.

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Note added in proof: A human lymphoma has recently been described which also expresses μ chain in the absence of light chains; "A human B-cell lymphoma synthesizing and expressing surface μ chain in the absence of detectable light chain". (Gordon, J., Hamblin, T. J., Smith, J. C., Stevenson, F. K. & Stevenson, G. T. *Blood* (in the press).)

Table 1 Differential responsiveness of 70Z/3 and 70Z/3.1 to LPS and DxS

Stimulus	70Z/3	% surface μ^+ 70Z/3-SF	70Z/3.1
—	2	0	0
LPS ($1 \mu\text{g ml}^{-1}$)	82	0	0
DxS ($50 \mu\text{g ml}^{-1}$)	76	73	76

Cell cultures were initiated at $2.5 \times 10^5 \text{ ml}^{-1}$ and exposed to either LPS or DxS for 24 h. Surface μ expression was determined using rhodamine-conjugated $[\text{F(ab')}_2]$ fragments of goat antibodies. For 70Z/3-SF cells, 70Z/3 cells were grown in serum-free Iscove's modified Dulbecco's medium (Gibco, No. 430-220) supplemented with lecithin/cholesterol, human transferrin (Hoechst) and delipidated bovine serum albumin (Behring, no. TR05) as previously described²⁰. The cells had been growing serum free for 6 weeks at the time of this experiment. For 70Z/3.1 cells, clones of 70Z/3 cells were obtained by initiating growth in RPMI medium (supplemented as described in Fig. 1 legend) containing 0.3% agar (Difco). Approximately 60–75% of the cells formed discrete colonies in these conditions. Clones were picked at day 6, transferred to microwells containing 0.2 ml medium and subsequently maintained in tissue culture flasks.

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Hormonal regulation of synthesis of yolk proteins and a larval serum protein (LSP2) in *Drosophila*

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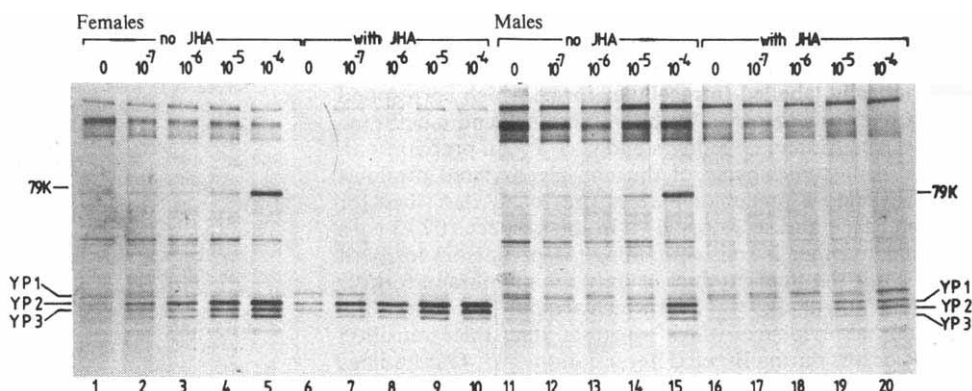
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Juvenile hormone (JH) and 20-hydroxyecdysone (20-HE) have a wide range of roles in insects. They interact during development to determine the quality of the moult^{1–4} and both hormones have been implicated in control of yolk protein synthesis and ovarian development. However, their functions vary in different insects^{5–7}. In adult *Drosophila*, they have specific effects on the haemolymph proteins. Both JH analogue (JHA) and 20-HE stimulate the synthesis and secretion of three yolk polypeptides (YPs) into the haemolymph of females^{8,9}. However, whereas JHA stimulates synthesis of YPs in both the fat body and the ovary, 20-HE stimulates the synthesis only in the fat body¹⁰, and JH, but not 20-HE, induces YP deposition in oocytes^{9,11,12}. 20-HE, but not JHA, evokes YP synthesis in *Drosophila* males (which do not normally produce these proteins¹³), and 20-HE treatment of isolated female abdomens stimulates incorporation of ³⁵S-methionine into a protein of molecular weight 79,000 (designated 79K). We have now compared the responses of the YPs and 79K to combinations of JHA and 20-HE given to both male and female *Drosophila* and show that 79K is a larval serum protein (LSP2) normally secreted by the fat body of third instar larvae. JHA and 20-HE have additive effects on YP synthesis, but JHA antagonizes 20-HE stimulation of LSP2 synthesis. This effect is probably caused by JHA induction of fat body cell death.

In an initial experiment, animals were ligated soon after eclosion, aged for 24 h to allow endogenous levels of hormone to decay and then treated in groups of 16 with increasing amounts of 20-HE in the presence or absence of JHA. After 6 h the haemolymph proteins were labelled by injecting ³⁵S-methionine and finally separated on SDS-polyacrylamide gel electrophoresis (PAGE). This experiment revealed three different effects of the hormone treatments. (1) Incorporation of label into YPs was stimulated additively by 20-HE and JHA in isolated female abdomens (Fig. 1, lanes 1–10, and Fig. 2), confirming earlier reports^{9,10}. (2) Secretion of newly synthesized YPs was also stimulated by 20-HE in isolated male abdomens

Fig. 1 JHA inhibits the 20-HE-stimulated label incorporation into 79K but augments that into the YPs. Isolated abdomens were prepared from newly eclosed flies by ligation with nylon thread between the thorax and abdomen, and removal of the head and thorax with scissors. After ageing for 24 h to allow the endogenous hormone levels to decline, abdomens were treated in groups of 16 with different concentrations of 20-HE in 1% ethanol/Ringer's solution. A second set of abdomens was treated with the same concentrations of 20-HE in the presence of a juvenile hormone analogue, ZR-515 (ref. 22) (0.3 μ l of an acetone solution of JHA was added topically to each abdomen giving a final dose of 1.6 ng ZR-515 per abdomen). After a 6-h incubation, the newly synthesized haemolymph proteins were labelled by injecting 1 μ Ci 35 S-methionine per abdomen in *Drosophila* Ringer²³. After a further 2-h incubation, the haemolymph was collected and mixed with sample buffer²⁴. One-quarter of each sample was electrophoresed on an SDS-containing 9–15% polyacrylamide gel²⁵; the Coomassie blue-stained gel was dried and autoradiographed. The figure is a photograph of the resulting autoradiograph. Lanes 1–10 and 11–20 are from female and male isolated abdomens respectively. Lanes 1–5 and 11–15 are from abdomens treated with increasing doses of 20-HE alone, while lanes 6–10 and 16–20 are from abdomens treated with increasing doses of 20-HE in the presence of JHA. 20-HE-stimulated label incorporation into YP1, YP2 and YP3 and the 79K polypeptide. Treatment with both hormones together stimulated label incorporation into YPs but not 79K.



(Fig. 1, lanes 11–15), demonstrating that the anterior of the male fly does not mediate the action of 20-HE. JHA alone had no effect in male abdomens (Fig. 1, lanes 11 and 16). Whereas the response to the hormones was approximately additive in females (Fig. 1, lanes 2–5 and 7–10), in males JHA made no consistent alteration to the 20-HE response (Fig. 1, lanes 12–15 and 17–20). (3) 20-HE stimulated synthesis of 79K in both males (Fig. 1, lanes 11–15) and females (Fig. 1, lanes 1–5), but this response was abolished in both sexes by the presence of JHA (Fig. 1, lanes 16–20 and 6–10, and Fig. 2). Thus in young adult haemolymph, the labelling of two simultaneously occurring groups of polypeptides is stimulated by 20-HE, and JHA adds to the 20-HE stimulation for one group of polypeptides but abolishes it for the other.

Because the nature and the site of synthesis of 79K were unknown, experiments were performed to identify the 79K polypeptide. During our work with YPs, we had noted that 79K was always seen in Coomassie blue-stained gels of haemolymph from newly eclosed males and females, but that it gradually disappeared from haemolymph of older animals (see Fig. 1 in ref. 10). This development programme for 79K coincides with that for the larval fat body. About 1,000 larval fat body cells survive metamorphosis but then histolyse in the first few days of adult life under the influence of JH^{14,15}. We thus suspected that 79K might be synthesized and secreted by the larval fat body cells that remain after eclosion. The major proteins synthesized and secreted by the larval fat body are the larval serum proteins (LSPs)¹⁶—LSP1, a hexamer containing combinations of three different polypeptides^{17,18}, and LSP2, a homohexamer¹⁹. As the four constituent polypeptides have estimated molecular weights of 75,000–83,000, it seemed reasonable that 79K seen in adult haemolymph at eclosion was a polypeptide constituent of LSP1 and/or LSP2 which was synthesized and secreted by the surviving larval fat body cells.

To test this hypothesis, 35 S-methionine-labelled haemolymph samples from abdomens which had been injected with either Ringer's solution or 20-HE were subjected to immunoelectrophoresis with antisera raised against purified LSP1 and LSP2 and compared with labelled haemolymph from mid-third instar larvae (Fig. 3). Larval haemolymph forms two major precipitin arcs, one corresponding to LSP2 and the other containing the three subunits of LSP1. Immunoelectrophoresis of the adult haemolymph showed that no label was incorporated into LSP1 in isolated abdomens irrespective of treatment with 20-HE. In contrast, LSP2 was heavily labelled in the haemolymph from the 20-HE-treated isolated abdomens and was weakly labelled in haemolymph from control abdomens.

To confirm that the arc formed with anti-LSP2 antisera indeed represented the 79K polypeptide identified by SDS-PAGE, a section of each precipitin arc was cut out of the agar plate and the sample run on SDS-PAGE with some of the original haemolymph samples. The labelled LSP1 precipitin arc from larval haemolymph separated as three distinct polypeptides but each of these was absent from the isolated abdomen haemolymph samples. The LSP2 precipitin arc was labelled in

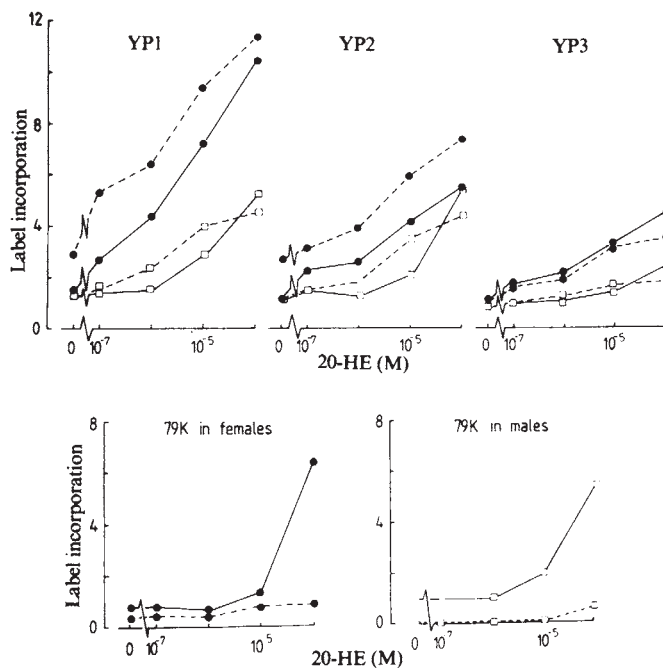


Fig. 2 Quantification of the response to hormone. Autoradiographs of the experiment in Fig. 1 were scanned using a Joyce-Loebl microdensitometer and the label incorporation estimated by measuring the peak areas of the 79K and YP bands. Each point represents the mean of two experimental replicates. Top panel, YPs; bottom panel, 79K. ●, Female abdomens; □, male abdomens. For both panels a broken line indicates JHA treatment with different concentrations of 20-HE; a solid line indicates 20-HE treatment in the absence of JHA. In females the two hormones stimulated label incorporation into the YPs additively, whereas JHA had no effect on the labelling of YPs in males. In both males and females 20-HE stimulated labelling of the 79K polypeptide but this effect was abolished by simultaneous treatment with JHA.

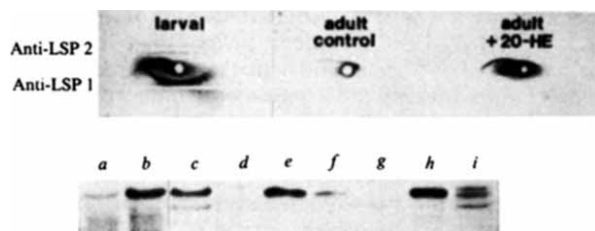


Fig. 3 79K is immunoprecipitated by antisera against LSP2. Labelled larval haemolymph was obtained by injecting mid-third instar larvae with ^{35}S -methionine dissolved in Ringer and collecting the haemolymph 2 h later. The adult haemolymph was obtained from abdomens which had been ligated at eclosion and allowed to age for 24 h. Controls were injected with Ringer and after 8 h injected with ^{35}S -methionine and haemolymph collected after a further 2 h. The 20-HE-treated abdomens were injected with 10^{-4} M 20-HE in 1% ethanol/Ringer 8 h before treatment with label. All haemolymph samples were mixed with unlabelled larval haemolymph, to act as carrier, and immediately subjected to immunoelectrophoresis. They were run in agar on microscope slides with 0.1 M sodium barbitone buffer (pH 8.4) at 80 V for 45 min at 4 °C. After electrophoresis each slot was filled with either anti-LSP1 or anti-LSP2 antisera, raised in rabbits from the purified proteins. After precipitation overnight the white precipitin arcs could be seen, and the agar was washed thoroughly in phosphate-buffered saline and stained with amido-Schwartz, dried and autoradiographed. The resulting autoradiograph is shown in the top panel. Larval haemolymph produced two main precipitin arcs corresponding to each of the LSPs. The adult haemolymph did not show any label incorporation into the LSP1 precipitin arc. A small amount of label was incorporated into the LSP2 arc of the control abdomens while considerably more was found in the haemolymph of those treated with 20-HE. Identical precipitin arcs were cut out of the agar without staining and mixed with SDS buffer before running on an SDS-containing 9% polyacrylamide gel. The gel was dried and autoradiographed. The 79K region of the resulting autoradiograph is shown in the lower panel: a, haemolymph from the control abdomens; b, haemolymph from abdomens treated with 20-HE; c, labelled mid-third instar larval haemolymph; d, LSP1 precipitin arc from 20-HE-treated abdomens; e, LSP2 precipitin arc from 20-HE-treated abdomens; f, LSP2 precipitin arc from control abdomens; g, LSP1 precipitin arc from control abdomens; h, LSP2 precipitin arc from larval haemolymph; and i, LSP1 precipitin arc from larval haemolymph. The LSP1 precipitin arc from larval haemolymph separates into the three LSP1 subunits but label is absent from this protein in the adult abdomens. The LSP2 precipitin arc is present in the haemolymph from the abdomens and label incorporation is stimulated by the treatment with 20-HE.

both adult haemolymph samples and co-migrated with LSP2 from larval haemolymph. In addition, the LSP2 from 20-HE-treated abdomens contained considerably more label than that from abdomens injected with Ringer alone.

These experiments have shown that both JHA and 20-HE act additively on YP synthesis. They also provide the first evidence that the appearance of LSP2 is also hormonally regulated, and that, in contrast with the YPs, its synthesis is stimulated by 20-HE only and abolished by JHA.

It follows from our observations that the larval fat body persisting in the newly eclosed adult is still capable of synthesizing LSP2. It also seems that the absence of LSP2 synthesis in the intact early adult is primarily because of the endogenous secretion of JH since merely ligating the abdomen causes a partial stimulation in synthesis of LSP2 and addition of JHA abolishes both this and the response to 20-HE. Once the source of JH has been removed by isolation of the abdomen, the larval fat body is able to synthesize LSP2 in response to 20-HE. Thus, although the effect has only been demonstrated at a developmental stage when the LSP2 gene is no longer active because of the presence of JH, it nevertheless suggests that the initial appearance of LSP2 may also be ecdysone induced. This is consistent with the observed ecdysone pulse at the second larval moult shortly before the appearance of LSP2 at the beginning of the third instar²⁰.

If 20-HE is indeed the hormonal trigger for LSP2 synthesis at the onset of the third larval instar, then either the titres of JH must be low or the larval fat body at that stage refractory to any naturally occurring JH. The latter is quite likely as larval fat body transplanted from third instar larvae into adults in contrast to larval fat body which has been through metamorphosis^{15,21}, is not susceptible to JH-induced histolysis. As the susceptibility of the larval fat body to JH changes during pupation, the inhibition

of the response to 20-HE could provide a means of monitoring the transition. The simplest explanation for the inhibitory action of JHA on the stimulation of LSP2 by 20-HE is that JH triggers cellular responses that lead to death of the remaining larval fat body¹⁵. However, this action of JH at the molecular level may be complex because the JH-induced death of the larval fat body is blocked by inhibitors of protein synthesis¹⁵.

Study of the appearance of LSP2 associated with the changes in the susceptibility of the larval fat body to these two major hormones should elucidate their actions on specific gene expression during development.

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A homoeotic mutation transforming leg to antenna in *Drosophila*

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Insects are thought to have evolved from millipede-like ancestors composed largely of a series of identical, leg-bearing segments. This view of insect evolution is supported by the existence of homoeotic mutations which transform particular abdominal and head segments into thoracic segments^{1–3}. Such transformations are described as atavistic³, because they return specialized segments to a more primitive condition. In *Drosophila*, several dominant mutations of the homoeotic locus *Antennapedia* (*Antp*) lead to a transformation of the antenna to the second leg^{4–8}. Here, I describe the isolation and characterization of apparent null alleles of the *Antp* locus. These mutations lead to a homoeotic phenotype which is the reverse of the dominant *Antennapedia* phenotype, namely, they result in the transformation of the second leg into an antenna but do not alter the development of the normal antenna itself. This result indicates that (1) the product of the wild-type *Antp* gene is normally active in the mesothorax where it promotes a mesothoracic pathway of development instead of an antennal pathway, (2) the *Antp*⁺ gene product is normally absent or inactive in the antenna, and (3) dominant mutations of the locus result in the inappropriate activity of the wild-type gene product in the antenna, and hence in the transformation of antenna to leg. Thus, unlike most other homoeotic gene products, the product of the *Antp*⁺ gene seems to promote, not to repress or modify, an atavistic condition.

Table 1 Homozygous *Antp*^{Ns+RC3} clones in the eye-antenna and mesothorax

Compartment	No. of blastoderm clones*	No. of first instar clones*	Phenotype†
Eye-antenna (A + P)	18	43	Normal
Leg 2 (A)	16(24)	18(24)	Antennal
Leg 2 (P)	14(15)	8(8)	Antennal
Notum + wing (A)	1(2)	9(16)	Abnormal

Marked clones of cells lacking the *Antennapedia*⁺ gene were obtained as follows. Embryos or larvae of the genotype *Antp*^{Ns+RC3} *e*¹¹/*Ki Sb*^{63b} *M(3)w*¹²⁴ were irradiated at appropriate times after egg laying (3 ± 0.7 = blastoderm stage; 24–48 h = first instar) (see Fig. 1 for description of mutations). All the mutations are located on the right arm of the third chromosome; because *Ki* is positioned closest to the centromere, mitotic recombinations which occur between *Ki* and the centromere can give rise to homozygous *Ki*⁺ cells which must also be homozygous for *Antp*^{Ns+RC3}, *Sb*⁺, *e*¹¹ and *M(3)w*⁺. 2,128 flies were screened following irradiation with 500 rad at the blastoderm stage, and 959 flies were screened following irradiation with 1,000 rad during the first larval instar. Clones lacking the *Antp*⁺ gene were identified under the dissecting microscope by the appearance of marked bristles and because they frequently disrupted the normal morphology. (Clones which mark, but do not transform, the distal portions of leg are difficult to identify under the dissecting microscope, and hence may have been missed.) All clones were mounted and inspected under the compound microscope. A comparable analysis of first instar clones of a second revertant allele, *Antp*^{Ns+RC11}, gave similar results.

*Number of clones in which some of the cells showed the normal, antennal or abnormal phenotype indicated in the last column (in those compartments in which some, but not all, of the clones were normal, the total number of clones is indicated in parentheses).

†Clones in the anterior compartment of the second leg were classified as antennal only when they formed antennal tissue. Clones in the posterior compartment of the second leg were classified as showing the antennal phenotype if they removed portions of the compartment—even when antennal tissue was not found (see text). The abnormal phenotype observed in notum clones is described in the text.

Mutations which eliminate most, or all, of the activity of the *Antp*⁺ gene were obtained using the general procedure of selecting phenotypic reversions of a dominant mutation⁹. This approach has been used by others^{10,11} to isolate apparent null alleles of the *Antp* locus. Homozygous *Antp*^{Ns} males were treated with the mutagen ethyl methane sulphonate (EMS)¹² and outcrossed to appropriately marked females. From among ~10,000 F₁ progeny, 35 phenotypic revertants were found; 12 of these progeny were found to carry 'revertant' alleles of the *Antp*^{Ns} mutation (designated *Antp*^{Ns+RC1, 2, 3, ... 12}). Three lines of evidence suggest that these revertant alleles eliminate most, or all, of the wild-type activity of the *Antp* locus. First, they arose with the expected frequency of null mutations in a single gene following a standard EMS mutagenesis¹³. Second, all these mutations are lethal over a deficiency of the *Antp* locus (*Df(3R)4Sch*; G. Jürgens, personal communication), as well as over other recessive lethal mutations of the locus (for example, *Antp*^B, *Antp*^{73b}, *Antp*^{Scx}). Third, animals which are homozygous or hemizygous for any of these reversions die as first instar larvae and show the characteristic terminal phenotype caused by loss of the wild-type *Antp* gene¹⁴ (but not those phenotypes associated with the loss of neighbouring genes, such as *Scr*, *R14*)¹⁴. It is possible that reversions of the *Antp*^{Ns} mutation may eliminate more than one genetic function; however, in the absence of substantive evidence that the *Antp* gene is itself complex, it is simplest to treat these reversions as mutations in a single gene.

The consequences of removing the wild-type *Antp* gene from particular cells during development have been studied by generating clones of cells homozygous for *Antp*^{Ns+RC} mutations in animals otherwise heterozygous for the mutation (see Fig. 1, Table 1). Cells belonging to these clones were independently marked using the bristle mutations *Kinked*, *Stubble*, *Minute(3)w*¹²⁴ and *ebony* (Fig. 1, Table 1; see refs 15 and 16 for descriptions of mutations). In addition, the *Minute* technique of

clonal analysis¹⁷ was used, so that clones of homozygous, *Antp*^{Ns+RC} *M(3)w*⁺ cells grew faster than surrounding, *Antp*^{Ns+RC}/*M(3)w*¹²⁴ cells, and hence formed large portions of the adult compartments^{18,19}. Clones were induced at the blastoderm stage as well as at later times during larval development. The results are shown in Fig. 1 and Table 1 and are described below.

First, clones in the eye-antennal segment were normal even when they were large enough to form most of the antenna, head capsule and maxillary palp (Fig. 1b, c). Similarly, clones in the proboscis, as well as at least the first six abdominal segments, were normal.

Second, clones in the anterior compartment of the second leg frequently transformed some parts of the leg into corresponding antennal structures (for example, distal portions of the leg, such as the tarsus, were transformed into tissue characteristic of the distal antenna, such as the third antennal segment (Fig. 1d–f)). However, only some of the marked cells showed the homeotic phenotype while the remaining marked cells formed normal second leg structures (Fig. 1d, e).

Third, clones in the posterior compartment of the second leg were generally associated with the absence of parts of the posterior compartment; in a few cases, vesicles of antennal tissue were also found associated with the clone (Fig. 1g). Note that the posterior compartment of the antenna is relatively small (Fig. 1c)²⁰. Consequently, a transformation of the posterior leg compartment into the posterior antenna compartment is expected to replace the leg compartment with only a small amount of antennal tissue.

Fourth, following irradiation at the blastoderm stage, the number of clones obtained in the anterior wing and notum (the dorsal derivatives of the mesothorax) were disproportionately small compared with the number of clones obtained in other appendages (Table 1). This result suggests that most blastoderm clones induced in the wing and notum primordium were lost during development or prevented the emergence of the flies bearing them. Wing and notum clones induced during the first larval instar and later, were found with similar frequencies to clones induced at the same time in other appendages. However, most of these clones showed abnormal patterns of notum bristles and in a few cases, atypical bristles were also observed.

Finally, clones induced during the blastoderm stage in the first and third legs were abnormal in about half the cases. Generally, such abnormal clones were associated with the absence of portions of the leg and with atypical cuticular patterns which could not be identified.

The principal result is that the phenotype of apparent null mutations of the *Antennapedia* locus (second leg transformed into antenna) is the reverse of that of dominant mutations of the

Table 2 Control of antennal versus second leg development by the *Antp*⁺ gene product

Genotype	Antenna		Second leg	
	<i>Antp</i> ⁺ gene product	Structure formed	<i>Antp</i> ⁺ gene product	Structure formed
Wild type	Absent	Antenna	Present	Leg 2
<i>Antp</i> ^{Ns/+}	(Present)	(Leg 2)	Present	Leg 2
<i>Antp</i> ⁻ / <i>Antp</i> ⁻	Absent	Antenna	Absent	Antenna

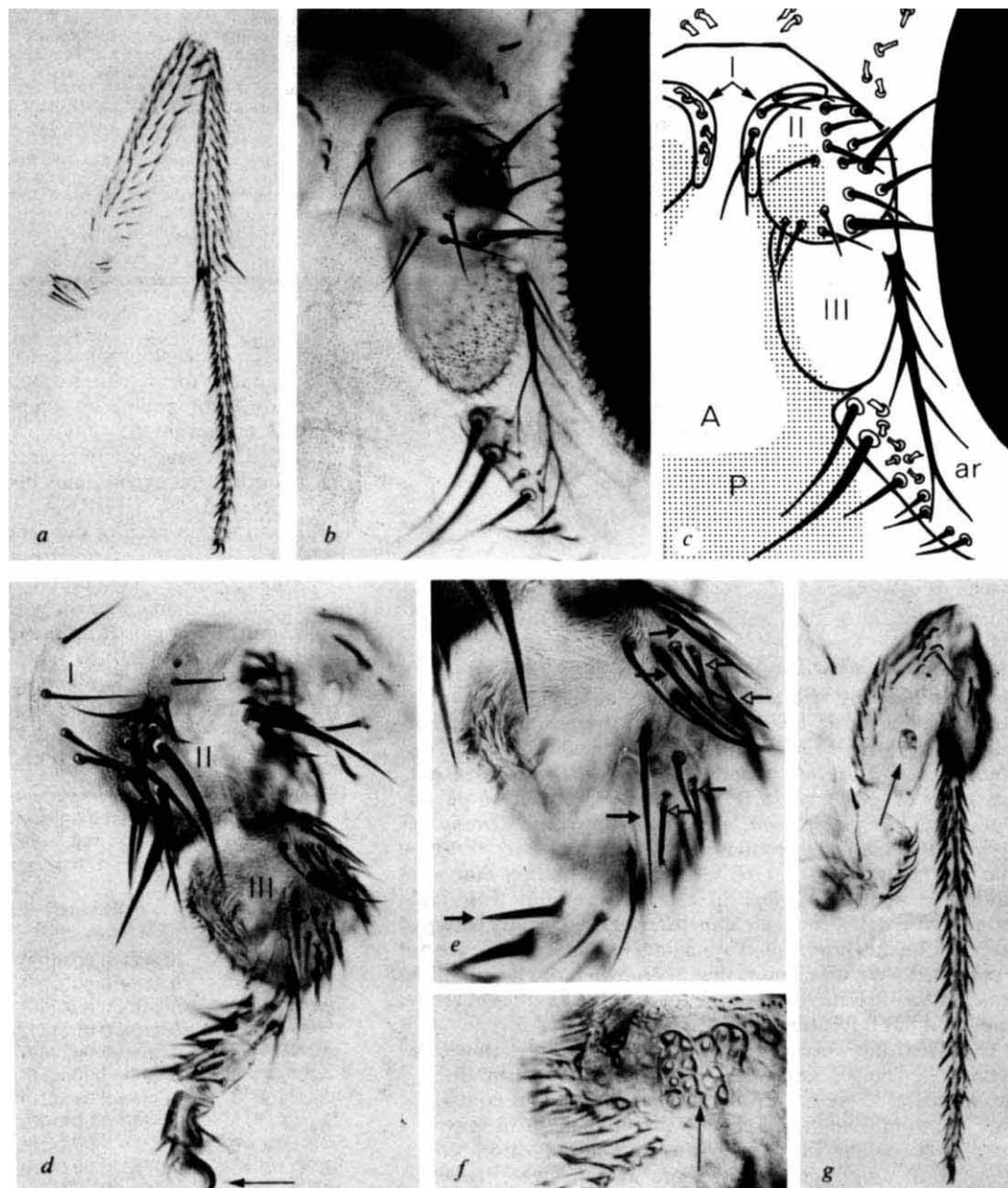
Model for the control of antennal versus second leg development by the *Antp*⁺ gene product. In both the antennal and second leg, presence (and activity) of the *Antp*⁺ gene product leads to leg development whereas absence of the active gene product leads to antennal development. Normally, the *Antp*⁺ gene product is absent (or inactive) in the antennal primordium, but present (and active) in the leg primordium. Mutations conferring the dominant *Antennapedia* phenotype result in the presence of active gene product in the antenna leading to an antennal to leg transformation (because this transformation is variable and incomplete it is likely that the gene product is only partially expressed or partially active in the antenna). Apparent null alleles of the *Antp*⁺ gene result in the absence of active *Antp*⁺ gene product in both the antenna and second leg, leading to a transformation of the leg into antenna.

locus (antenna transformed into second leg). This situation parallels that of dominant and recessive mutations of several of the genes of the *bithorax* complex¹, and leads to the following conclusions: (1) in wild-type flies, the *Antp*⁺ gene product is normally active in the mesothorax where it promotes mesothoracic as opposed to antennal development, but absent or inactive in the eye-antennal segment, and (2) mutations of the locus which confer a dominant *Antennapedia* phenotype result in inappropriate activity of the *Antp*⁺ gene product in the antenna, thereby transforming the antenna into a second leg (Table 2). These conclusions must be considered in the light of the homoeotic phenotypes of embryos lacking the *Antp*⁺ gene. Wakimoto and Kaufman¹⁴ have described this phenotype as a partial transformation of the meta- and mesothorax into prothoracic segments, and concluded that the *Antp*⁺ gene is normally required in the meso- and metathorax for promoting a normal as opposed to a prothoracic pathway of development. If

their interpretation of the embryonic phenotype is correct, the results presented here would indicate that the *Antp*⁺ gene product controls one developmental alternative (mesothorax instead of prothorax) in embryonic cells giving rise to the larval cuticle, and another developmental alternative (mesothorax instead of eye-antenna) in imaginal cells giving rise to the adult cuticle.

A second result of interest is that the leg-to-antenna transformation observed in clones lacking the *Antp*⁺ gene is shown by some, but not all, of the cells belonging to the clone, the remaining cells forming normal second leg structures. Although there are many possible explanations for this apparent non-autonomy, the simplest is that the wild-type *Antp* gene product can pass between cells, thereby allowing heterozygous cells to rescue neighbouring mutant cells. This interpretation is supported by the observations that large clones generally formed antennal structures whereas small clones did not (data not

Fig. 1 Phenotype of somatic clones lacking the *Antennapedia*⁺ gene in the antenna and second leg. *a*, Wild type, second leg $\times 56$. *b*, *c*, Fly's head bearing a marked clone induced at the blastoderm stage which forms a normal antenna and part of the head capsule. Cells of the clone are marked by the recessive mutation *ebony*¹¹ (*e*¹¹) which darkens the normal colour of cuticular structures, particularly bristles; cells outside the clone are marked by the dominant mutations *Kinked* (*Ki*), *Stubble*^{63b} (*Sb*^{63b}) and *Minute(3)*_w¹²⁴ (*M(3)*_w¹²⁴) which together result in short, stunted bristles (see refs 15, 16 for descriptions of mutations). In *c*, bristles belonging to the clone are blacked in, whereas bristles outside the clone are drawn in outline. The three antennal segments (I, II and III), the arista (ar) and the anterior (A; unshaded) and posterior (P; shaded) compartments of the eye-antenna are indicated in *c*. $\times 186$. *d*, Blastoderm clone which forms almost the entire anterior compartment of the second leg, transforming it into corresponding portions of the antenna. Note the presence of structures characteristic of the first (I), second (II) and third (III) antennal segments. Note also that marked cells situated at the margins of the clone form normal leg tissue (in this case, a claw (arrow), and bristles characteristic of the distal leg). The untransformed posterior compartment lies beneath the transformed anterior compartment and hence is out of the plane of focus. $\times 224$. *e*, Detail of *d* showing apparent non-autonomy of the leg-to-antenna transformation. Marked cells at the periphery of the clone form bracted bristles characteristic of the distal leg (filled arrows indicate bristles formed by the clone, outlined arrows indicate bristles formed by cells outside the clone; bracts can be seen as small spikes at the base of most leg bristles. $\times 352$. *f*, Detail of the antennal tissue in *e* showing an antennal sense organ, the sacculus²³ (arrow), formed inside the homoeotic antenna. $\times 520$. *g*, Second leg bearing a blastoderm clone which forms virtually the entire posterior compartment. Although the most proximal and most distal portions of the posterior compartment are not transformed (even though marked by the clone), the middle portions of the compartment are absent, giving the leg a truncated appearance (compare with *a*). Note also a vesicle of antennal tissue within the leg (arrow). $\times 96$.



shown), and that mutant cells which were untransformed were generally positioned at the periphery of mutant clones, next to heterozygous cells (for example, Fig. 1e). In this context, it is interesting that regions of the leg such as the distal tarsus and claw which were never transformed (Fig. 1d) are situated next to the anteroposterior compartment boundary²¹, and hence, are always adjacent to heterozygous cells which may rescue the mutant phenotype. The possibility that the wild-type *Antp* gene product can pass between cells also provides a plausible explanation for the results of Kauffman and Ling²², who found that wild-type (+/+) clones in the antennae of *Antp^{Ns}/+* flies often formed leg tissue.

Studies of genes of the *bithorax* complex have provided support for the supposition that a series of thoracic-like segments was progressively modified during evolution to form the sequence of specialized segments of the insect abdomen and thorax¹. If a similar process modified more anterior, thoracic-

like segments into the specialized segments of the head, it is possible that a small number of control genes may be responsible for specifying the development of the head segments. Clearly, the products of such head promoting genes should not function in the thoracic segments, otherwise they also would develop into head segments. It is possible, therefore, that the product of the *Antp*⁺ gene normally acts in the thorax to repress the function of head promoting genes, but is absent (or inactive) in the head segments where the functions of these genes are required for normal development.

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Intervening sequences in ribosomal RNA genes and *bobbed* phenotype in *Drosophila hydei*

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The '*bobbed*' (*bb*) mutation in *Drosophila* is represented phenotypically by shortened and abnormally thin scutellar bristles and by delayed development. There is a direct correlation between bristle size and ribosomal RNA (rRNA) synthesis¹, and the *bb* mutation was at first explained as a deficiency of rRNA genes (rDNA)². However, the *bb* phenotype can occur in *Drosophila melanogaster*¹ and *Drosophila hydei*³ with high rDNA content, while phenotypically wild-type flies are known with few rRNA genes, suggesting that what matters is not the number of rRNA genes but their transcriptional activity. In *D. melanogaster*, it has recently emerged that rRNA genes interrupted by an intervening sequence are not transcribed⁴. We now report that in *D. hydei*, the length of the scutellar bristle is directly proportional to the number of rRNA genes without this intervening sequence.

D. hydei has three clusters of rRNA genes (nucleolar organizers, NOs), one on the X and two on the Y chromosome^{5,6}. We have previously described flies containing only Y-chromosomal NOs which were not *bobbed* in spite of a low rDNA content. In contrast, flies with only X-chromosomal NOs, containing significantly more rDNA, were *bobbed*³. Clearly in *D. hydei*, the NOs on the Y and X chromosomes do not have a comparable influence on the *bobbed* phenotype.

Many X-chromosomal rRNA genes in *D. hydei* contain an intervening sequence 6 kilobases (kb) long (*ivs*⁺ genes)⁷, but in several genotypes with only Y-chromosomal NOs, no *ivs*⁺ rRNA genes have been detected. As in *D. melanogaster*, *ivs*⁺

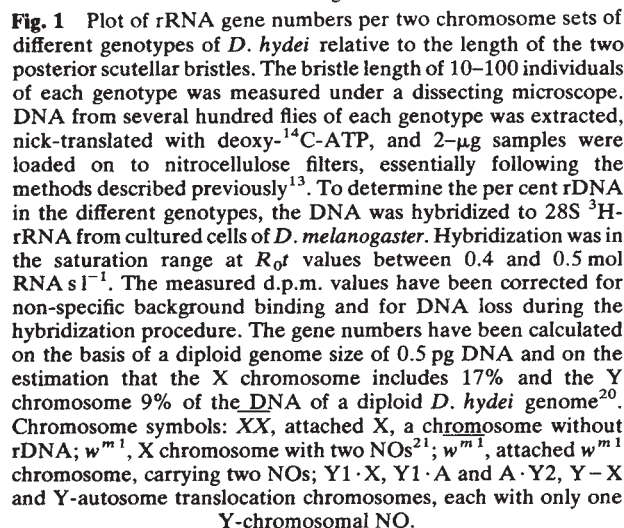
genes in *D. hydei* do not seem to be significantly transcribed⁸. Consequently, the X-chromosomal NO, in contrast with the Y-NO, does not seem to be completely functional with respect to rRNA transcription.

Total rRNA gene numbers were determined by filter saturation hybridization experiments between ¹⁴C-DNA from whole adult flies and 28S ³H-rRNA. To eliminate variation of *bb* expression within each of our laboratory strains, each investigated strain was newly started from a single pair mating, and flies from the F₂ or F₃ generation used. Among 20 genotypes investigated, 11 had either the wild-type scutellar bristle length of 0.57 mm or very close to it, in spite of the fact that their rDNA content varied from 210 to 1,113 genes per two chromosome sets (Fig. 1, Table 1). Two genotypes showed slightly reduced bristle lengths (0.50 and 0.52 mm), although their rRNA gene number was very high (780 and 1,067). Flies from seven other genotypes were phenotypically clearly *bobbed*, with bristle sizes

Table 1 Bristle length and number of *ivs*⁻ and *ivs*⁺ rRNA genes per two chromosome sets from *bb* and wild-type flies

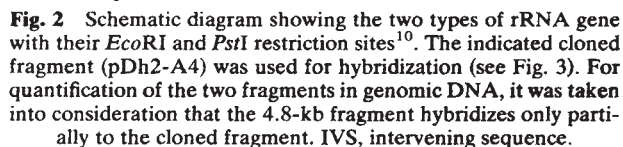
Genotype	Bristle length ± s.e.m. (n)	rRNA gene number ± s.e.m.		
		<i>ivs</i> ⁻	<i>ivs</i> ⁺	F; G
<i>X^{bb1}/O</i>	0.212 ± 0.004 (33)	28 ± 4	189 ± 8	18; 3
<i>X^{bb1}/X^{bb1}</i>	0.316 ± 0.002 (31)	55 ± 6	365 ± 14	18; 3
<i>X^{bb2}/O</i>	0.306 ± 0.004 (10)	70 ± 7	456 ± 13	40; 4
<i>X^{bb3}/O</i>	0.240 ± 0.002 (21)	52 ± 6	388 ± 13	14; 3
<i>X^{bb4}/O</i>	0.305 ± 0.002 (69)	80 ± 6	341 ± 13	23; 4
<i>X^{bb4}/X^{bb4}</i>	0.495 ± 0.006 (25)	135 ± 9	645 ± 20	19; 3
<i>X^{bb5}/O</i>	0.354 ± 0.006 (10)	94 ± 5	422 ± 15	30; 4
<i>w^{m1}/w^{m1}</i>	0.518 ± 0.004 (30)	142 ± 14	925 ± 30	30; 3
<i>w^{m1}/O</i>	0.335 ± 0.002 (100)	70 ± 6	851 ± 31	28; 3
<i>X/O</i>	0.568 ± 0.002 (39)	220 ± 17	151 ± 16	27; 3
<i>X/X</i>	0.554 ± 0.003 (20)	428 ± 15	276 ± 12	23; 3

n, Number of flies of which the two posterior scutellar bristles have been measured. F, number of filters which have been measured per genotype to determine the rDNA percentage. G, number of gels used for Southern transfer, hybridization and determination of the *ivs*⁻/*ivs*⁺ rRNA gene relationship.



When only the *ivs*⁻ gene number of each genotype is plotted relative to the bristle length, a direct proportionality between the gene number and phenotypic *bobbed* expression is evident in the range between 30 and ~150 *ivs*⁻ genes (Fig. 4). The correlation coefficient in this range is 0.95. The *bobbed* phenotype is

Because gene compensation in *D. hydei* has hitherto been only observed with Y-chromosomal NOs^{13,16}, we selected genotypes with only X-NOs for our investigations. It is less easy to exclude independent rDNA polyploidization, because this process occurs with both Y- and X-chromosomal NOs¹³. For example, the *ivs*⁻ rDNA percentage in whole X/O males is almost exactly half that in X/X females (Table 1), but in salivary glands the *ivs*⁻ rRNA gene numbers of both genotypes were the



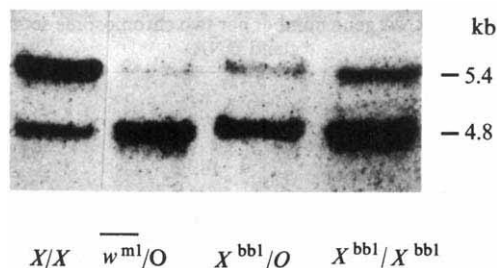


Fig. 3 Autoradiogram of *EcoRI*-*PstI*-digested DNA hybridized with the cloned fragment pDh2-A4 (ref. 10) (see Fig. 2), demonstrating different *ivs*⁻/*ivs*⁺ gene relationships in four genotypes. In this experiment, 1 µg of nuclear DNA from whole adult flies of each genotype was loaded on 1% agarose gels, transferred to a filter and hybridized. The hybridization conditions used were: (1 µg cloned DNA in 10 ml 2×SSC (1×SSC=0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 0.2% SDS, 1× Denhardt's solution; 40 h. The measured relationship of the two fragments within the genotype was independent of the amount of input DNA. For tracing on the Joyce-Loebl Chromoscan, autoradiograms were used only where the film density was linear with increasing exposure time.

same (Table 2). If such independent rDNA polyploidization also occurs in genotypes with *bobbed* X chromosomes, the difference in bristle length between *X/O* and *X/X* constitutions measured in *bobbed-1* and *bobbed-4* stocks (Fig. 4) is difficult to understand.

As it is very difficult to determine gene numbers directly on bristle-forming cells, we used a representative polyploid tissue, salivary glands, to determine the *ivs*⁻ rRNA gene numbers in our *bobbed-4* stock. We found that *X^{bb4}/X^{bb4}* females have twice as many *ivs*⁻ rRNA genes per chromosome set as *X^{bb4}/O* males (Table 2). Thus the *bobbed-4* X chromosome differs from the wild-type X chromosome by being replicated proportionally during polyploidization with respect to its *ivs*⁻ rRNA genes. The *bobbed* chromosomes in the other stocks probably behave similarly, so that *bobbed* flies with low *ivs*⁻ rRNA gene numbers also have low numbers in their polyploid tissue.

Note that none of our investigated genotypes has a low number of both types of rRNA gene (Table 1). Genotypes with low *ivs*⁻ gene numbers have relatively high percentages of *ivs*⁺ genes (81–92%), whereas the wild type has only 40% *ivs*⁺ genes. It is possible that the two types of rRNA gene change their degree of multiplicity in a dependent manner; distinct loss in *ivs*⁻ genes (resulting in the *bobbed* phenotype) seems to be accompanied by an expansion of *ivs*⁺ genes. Further experiments are being done to investigate this possible correlation in stocks where spontaneous *bobbed* mutations occur. Such a correlation would explain the observation¹⁷ that the *bobbed* mutation in *D. hydei* is almost exclusively associated with the

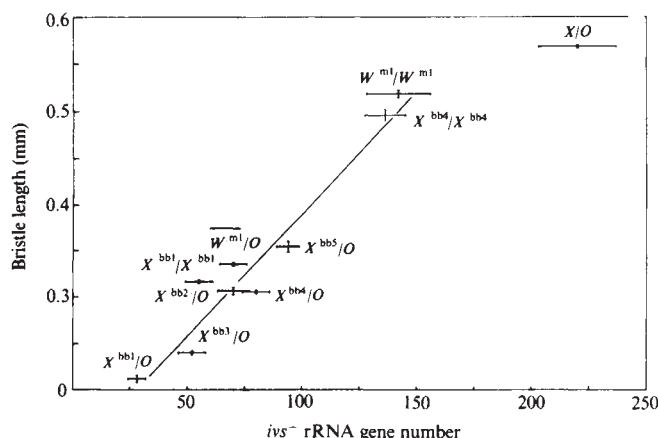


Fig. 4 Direct correlation between *ivs*⁻ rRNA gene number per two chromosome sets and the length of the two posterior scutellar bristles of the same genotypes as in Table 1.

X-chromosomal NO. Only two *bobbed* mutants of the Y chromosome have hitherto been recorded¹⁸. In contrast, in *D. melanogaster bobbed* Y chromosomes are frequent². In this species, *ivs*⁺ genes also occur in the Y-chromosomal NO, whereas in all genotypes hitherto investigated⁷ in *D. hydei*, the Y-NOs seem to be completely free of *ivs*⁺ rRNA genes.

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Multiple copies of iso-insertion sequences of IS1 in *Shigella dysenteriae* chromosome

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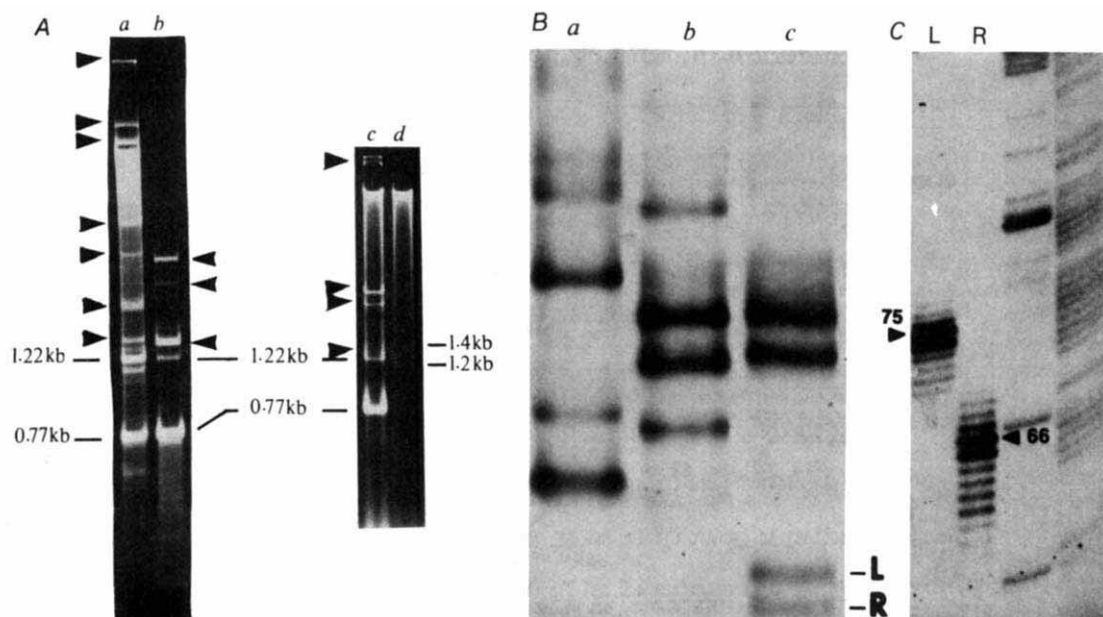
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The insertion sequence, IS1, which is 768 base pairs (bp) long¹, is the smallest active translocatable DNA element in bacteria (for review see ref. 2), and appears as a repeated DNA sequence in some Enterobacteriaceae species³. In particular, most *Shigella* species contain more than 40 copies of a sequence that hybridizes to IS1³. Here, we report the isolation of various repeated sequences from the *Shigella dysenteriae* chromosome and the DNA sequence analysis of the class, which is about 770 bp long. The results demonstrate that there are two major types of sequence in this class; one is identical to IS1 except for 10-bp substitutions and is present in about 50 copies in the chromosome; the second sequence, 766 bp long, is repeated more than 150 times and has ~55% homology to IS1 on the nucleotide sequence level. Because of this high degree of homology, we call these 'iso-insertion sequences' of IS1. Further examination of the nucleotide sequence reveals common coding frames between these sequences, suggesting the existence of two genes that are required for multiplication of these sequences.

Examination of *Sh. dysenteriae* DNA by electron microscopy after denaturation and renaturation revealed many single-stranded loop structures with duplex stems, indicative of the presence of inverted repeat sequences⁴. Of the 70 duplex stems examined, 44 formed a distinguishable peak at 0.77±0.06 kilobases (kb) in the histogram of length versus number of molecules. This size category of inverted repeat sequence comprised 3.94% of the total DNA strands measured. Assuming that the *Sh. dysenteriae* chromosome is the same size as that of *Escherichia coli*, namely 2.5×10⁹ molecular weight (*M_r*) or 4×10⁶ kb (ref. 5), the estimated number of copies of these inverted repeat sequences is ~200 per chromosome.

We then used an S₁ nuclease digestion technique to purify the inverted repeat sequences on the chromosomes (see Fig. 1 legend). Figure 1A shows the repeated sequences purified from

Fig. 1 A, 1.4% agarose gels, showing bands of duplex DNA isolated from *Sh. sonnei* (a), *Sh. dysenteriae* (b, c) and *E. coli* K-12 (W3110) (d) chromosomes. The duplex DNAs were prepared as follows. The chromosomal DNA was first isolated by the method described by Saito and Miura⁶, dissolved in 10 ml of TE buffer containing 0.01 M Tris (pH 7.2) and 0.001 M EDTA, titrated to pH 12.3 with 2 M NaOH at room temperature and further incubated for 15 min. The solution was then neutralized with 2 M HCl to pH 7.0. The Na⁺ concentration in the DNA solution was adjusted to 0.3 M with 3 M NaCl and the solution incubated at 65 °C for 1 min for renaturation of inverted DNA sequences on the DNA strands. The single-stranded DNA in the solution was digested at 37 °C for 1 h with 50 U per µg DNA of S₁ nuclease in a



buffer containing 40 mM sodium acetate (pH 4.6) and 4.5 mM ZnCl₂. After extraction of the solution with phenol saturated with TE buffer, the DNA was precipitated with ethanol and then suspended in 1 ml of TE buffer. 7 ml of 0.12 M sodium-potassium phosphate buffer (Na-P buffer, pH 6.8) containing 2 ml of hydroxyapatite (HAP) suspension (Bio-Rad) was added to the DNA solution and incubated at 65 °C for 30 min. The solution was occasionally stirred with a glass rod to enhance the efficiency of adsorbing double-stranded DNA. The HAP-DNA complex was precipitated by centrifugation and resuspended in the Na-P buffer and the adsorption step above was repeated three times. Double-stranded DNA was then eluted from the HAP in 5 ml of 0.4 M Na-P buffer (pH 6.8) by incubation at 65 °C for 30 min. The elution step was repeated and a total of 10 ml of eluate saved. The solution was concentrated to 2 ml in dialysis tubing which was embedded in polyethylene glycol powder (Carbowax 6000, Bio-Rad), followed by dialysis against TE buffer. After precipitation of the DNA with ethanol, the DNA was resuspended in 200 µl of TE buffer. 10 µl of the solution was used for gel electrophoresis. The above procedure also extracts the plasmid duplex DNAs, present in the bacterial cells. The bands corresponding to plasmid DNA linear, open and closed circular configurations are shown by solid triangles. B, autoradiographs of 6% polyacrylamide gels showing 5'-³²P-labelled fragments of purified inverted repeat DNA sequences of the *Sh. dysenteriae* chromosome which were cleaved with *Hae*II (a), *Hpa*II (b) and *Alu*I (c), respectively. C, autoradiographs of 10% polyacrylamide-7 M urea gel containing the two small 5'-³²P-labelled *Alu*I fragments, R and L, which were denatured with alkali and heated at 95 °C after elution of DNA bands shown in Bc. The ladders of bands (differing by one nucleotide in size) suggest that the 5' ends of these fragments are actually heterogeneous. (Ladders seen in two other lanes on the right are size markers that were obtained by sequencing a 5'-³²P-labelled fragment by the method of Maxam and Gilbert⁷.) When the DNA in each of the most intense bands in the autoradiogram was eluted from the gel and subjected to nucleotide sequence analysis, the sequences at the two ends of *IS1D* were almost identical to those at the ends of *IS1R* (see Fig. 3).

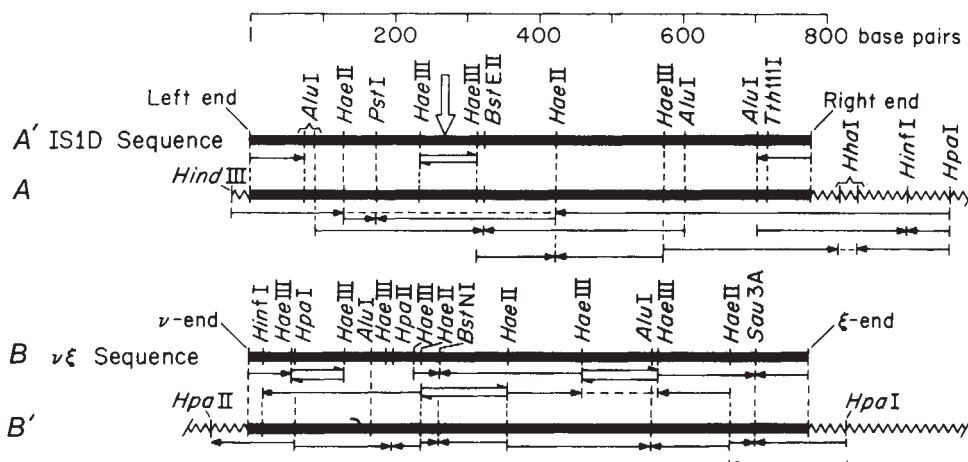
Sh. dysenteriae, *Shigella sonnei* and *E. coli* K-12 chromosomal DNA after gel electrophoresis. It is clear that all produce a number of gel bands similar in size to known insertion sequences such as *IS1*, *IS2* and *IS3*. Note, however, that *Sh. dysenteriae* (also *Sh. sonnei*) produces an extremely dense band of DNA about 770 bp long.

We purified the 770-bp sequences of the *Sh. dysenteriae* chromosome, labelled the 5' ends of the DNA with ³²P and cleaved with various restriction endonucleases. As shown in Fig. 1B, electrophoresis and autoradiography showed two pairs of bands, suggesting that DNA from the 770-bp region is composed of two major sequences that yield different cleavage patterns for the enzymes used. Ethidium bromide staining of these digests revealed two major groups of gel bands with

different intensities (data not shown). The fragments from each group had a sum molecular length of about 770 bp. The ratio of the two components was estimated as 1:3 from the autoradiogram.

Restriction mapping revealed only a single difference between the minor 770-bp sequence and the *IS1* sequenced by H.O. and E.O.¹. A *Hae*III site absent in the minor fragment (we named this *IS1D*) was present in *IS1* (ref. 1) (we now call this *IS1R*) (see Fig. 2A). However, the map of the major 770-bp component (which we call *νξ* in order to assign an orientation to this sequence) was completely different (see Fig. 2B). The same results were obtained using restriction fragments of the *Sh. dysenteriae* chromosomal DNA which had been cloned into the plasmid pBR322 (see Fig. 2).

Fig. 2 A and A' show cleavage maps of *IS1D* purified by S₁ nuclease and of *IS1D* present in a *Hind*III fragment cloned into pBR322, respectively. In A', the *Sh. dysenteriae* unique sequence is shown by sawtooth lines. Note that a *Hae*III site at the position indicated by a vertical open arrow is present in *IS1R* but is absent in *IS1D*. B and B' show cleavage maps of the *νξ* sequence purified by S₁ nuclease and of that isolated as a *Bam*HI fragment cloned into pBR322, respectively. In B' the sawtooth lines represent the unique sequence of the *Sh. dysenteriae* chromosome. The numerous horizontal arrows indicate the strategy used for determining the nucleotide sequence of *IS1D* and *νξ*.



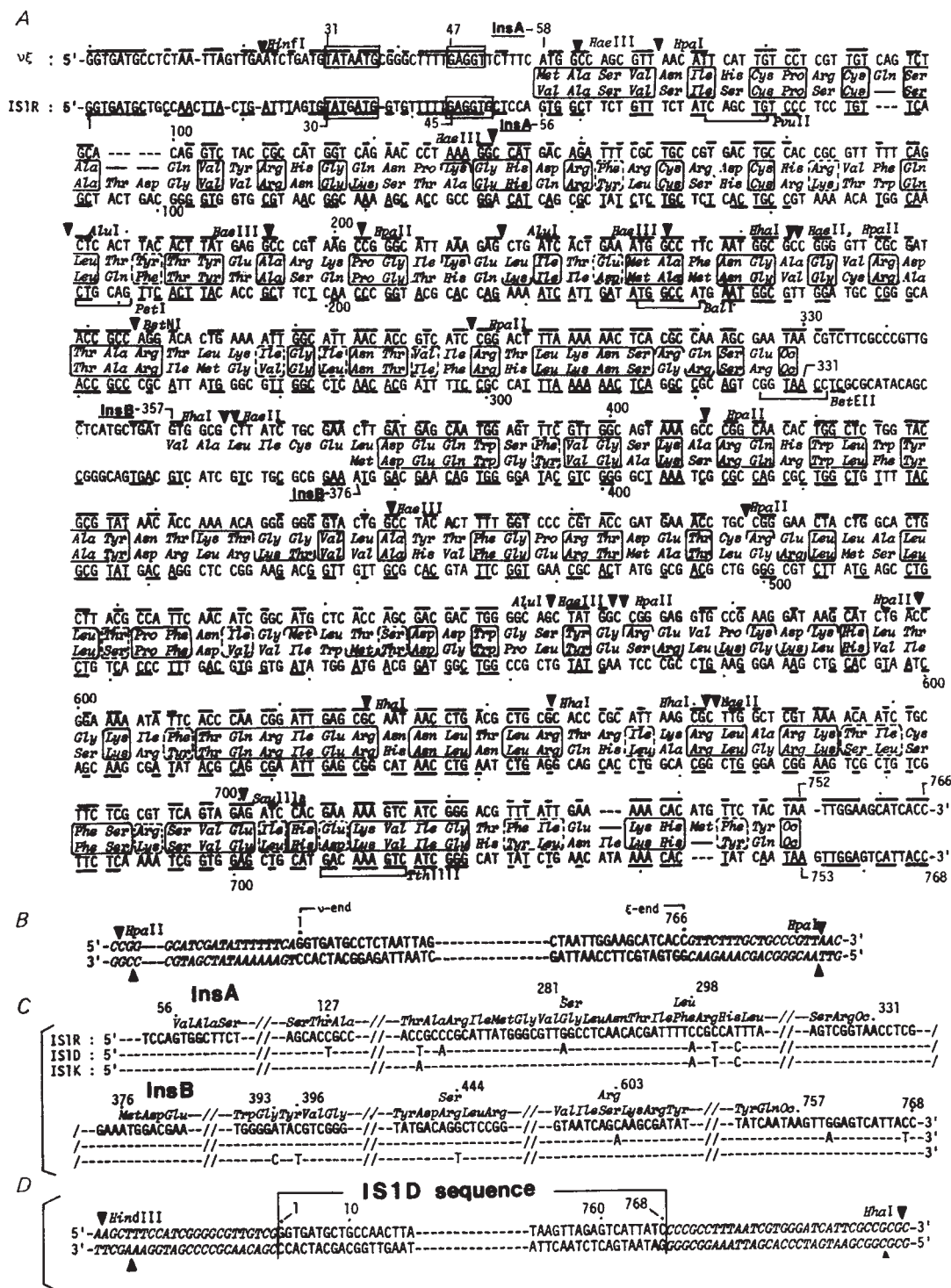


Fig. 3 A, the entire nucleotide sequence of the *vξ* sequence determined by the method of Maxam and Gilbert⁷. The sequence is aligned with the nucleotide sequence of ISIR, which has previously been determined, to indicate the sequence homology between them (see lines on the common nucleotides between them). The two sequences encode two common frames, *insA* and *insB*, coding for hypothetical amino acid sequences as shown. Homologous amino acids are boxed. B, a part of *vξ* and unique chromosomal sequence (italic letters) in the fragment cloned into pBR322. C, critical parts of the ISIR sequences together with the amino acid sequences of *insA* and *insB*. Nucleotide sequences of ISID and ISIK differ from ISIR as shown. Some base substitutions cause amino acid changes as indicated. D, a part of the ISID sequence as well as a unique chromosomal sequence (italic letters) of the *Sh. dysenteriae* chromosome which has been cloned into pBR322.

Different regions of the *vξ* sequence were labelled with ³²P and used as probes to identify *vξ* in the chromosomes of various *Shigella* species and the *E. coli* K-12 strain, JE5519, using the Southern blotting and hybridization techniques that we have previously used⁷. The *vξ* probes hybridized with many *EcoRI* restriction fragments of *Sh. dysenteriae* DNA, but the pattern of these fragments was completely different from that previously seen when an ISIR probe was used. Also, the *vξ* probe hybridized with only one *EcoRI* restriction fragment from *Shigella flexneri* and *Shigella boydii* and none from *Sh. sonnei* or JE5519

(data not shown), although several restriction fragments of these four species hybridized to ISIR³. These results suggest that *vξ* does not cross-hybridize with sequences which hybridize to ISIR probes.

We next used the Maxam and Gilbert method⁷ to determine the entire nucleotide sequence of *vξ* and ISID isolated by the S₁ nuclease technique and by cloning into pBR322. The strategy used for sequencing is shown in Fig. 2. Sequencing the ends of the fragments purified using S₁ nuclease presented some difficulty, because the 5' ends were found to be heterogeneous

(see Fig. 1C and its legend). Figure 3A shows the entire nucleotide sequence of $\nu\xi$ purified by the S_1 nuclease technique. The nucleotide sequence of the cloned copy of $\nu\xi$ gave the same result, suggesting that the repeated sequences of this kind in the *Sh. dysenteriae* chromosome are homogeneous. Although the cleavage map of the $\nu\xi$ sequence was completely different from that of IS1R (and IS1D), the nucleotide sequence of $\nu\xi$ showed 55% homology with that of IS1R as represented in Fig. 3A. We also found that a 31-base sequence at the ν end of the $\nu\xi$ sequence appeared in an inverted orientation at the ξ end, although there are 6 base mismatches between them. Small inverted repeat sequences are also present in IS1R^{1,8-10} and are quite homologous to those at the ends of $\nu\xi$.

When the $\nu\xi$ and IS1R sequences were searched for possible coding frames, two common frames, which we call *insA* and *insB*, were present (Fig. 4). They code for polypeptides of very similar size (*InsA*, $M_r \sim 10,000$, and *InsB*, $\sim 15,000$) and their amino acid sequences are remarkably similar (see boxed region in Fig. 3A). If pairs of related amino acids (for example, Arg-Lys) are considered to be equal, the two polypeptides from $\nu\xi$ show about 56% homology to those from IS1R.

The nucleotide sequence analysis of IS1D showed only 10 base pair changes between IS1R and IS1D. As shown in Fig. 3C, only two of these substitutions in *insA* and one in *insB* cause amino acid changes.

It has been reported that the nucleotide sequence of an IS1 present in the *E. coli* K-12 chromosome shows seven base pair changes when compared with that of IS1R⁸. In this IS1, which we refer to as IS1K, one of the base changes causes an amino acid change in polypeptide *InsA*, another changes an amino acid in *InsB* (Fig. 3C).

We postulate that the two coding frames, *insA* and *insB*, and the inverted repeat sequences seen at the ends of the $\nu\xi$ and IS1D sequences are necessary for translocation of IS1 and its iso-insertion sequences. In fact, genetic studies in our laboratory show that deletion mutations in either of these two coding frames or in the two ends of IS1R cause loss of translocation activity^{11,12}.

In the region preceding the *insA* coding frame, but not the *insB* coding frame, we identified both a ribosome binding (Shine-Dalgarno) sequence¹³ (nucleotides 47–51 in $\nu\xi$ and 45–50 in IS1R) and a –10 homology region (or Pribnow box)^{14,15} (nucleotides 31–37 in $\nu\xi$ and 30–36 in IS1R) as indicated in Fig. 3A. This suggests that the *insA* message is probably transcribed and translated efficiently. On the other hand, the lack of obvious promoter sequence in the region preceding *insB* suggests either that *insB* may be transcribed very inefficiently or that it is transcribed as a part of a polycistronic message beginning at the *insA* promoter. The fact that the region preceding *insB* also lacks a Shine-Dalgarno sequence may suggest, however, that *insB* is very poorly translated even when the polycistronic message is transcribed efficiently. The presumed inefficient translation of *insB* may be related to control of the rate at which IS1 multiplies in the chromosome.

The nucleotide sequences of unique chromosomal DNA adjacent to both ends of the $\nu\xi$ sequence and IS1D in the cloned fragments are shown in Fig. 3B and D. Although it is generally accepted that insertion sequences and transposable elements generate directly repeated duplications of several bases at the target site on translocation (for review see ref. 2), no such direct repeat sequences are present at the junctions of $\nu\xi$ and IS1D.

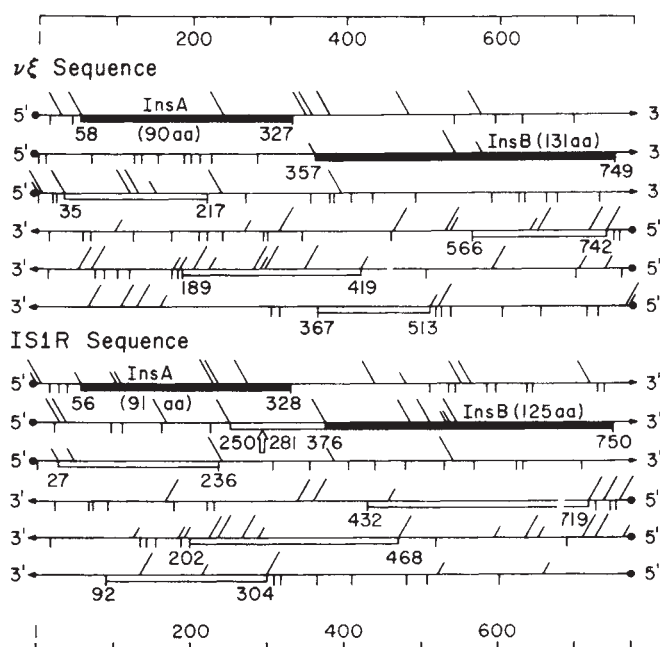


Fig. 4 The possible initiation and termination codons formed within $\nu\xi$ and IS1R. All three reading frames of both strands were analysed as shown. Long angled lines represent AUG start codons; short angled lines represent GUG start codons. Vertical lines indicate the presence of a UGA, UAA or UAG termination codon. All open frames for possible polypeptides are indicated in positions with numbers (see Fig. 3). The two open frames common to the two sequences are labelled *InsA* and *InsB* as shown. Note that the base change in IS1D at position 281 (which eliminates a *HaeIII* site present in IS1R) introduces a termination codon into an open frame which precedes *insB* of IS1R (see open arrow), indicating that the open frame from 250 to 376 is not essential for translocation of IS1R or IS1D.

The absence of direct repeat sequences could be explained in two ways. First, deletions which occurred at the precise ends of the insertion sequences after the translocation of these sequences would result in loss of the part of the chromosome that includes the duplicated sequence. Alternatively, two copies of these repeated sequences flanking a unique chromosomal sequence could become a transposable element [as has been observed for transposable elements such as *Tn9* and *Tn1681* (refs 16–18)] and move into other sites on the chromosome. In these cases, the individual IS1 components of the transposable element are not flanked by direct repeats^{1,19}.

IS1 is not thought to be essential for the survival of bacterial cells. Nevertheless, it can multiply within a bacterial host, resulting in large numbers of IS1 sequences within the host chromosome. Also, the various types of base changes that we have observed in iso-insertion sequences suggest that IS1 can evolve independently within a bacterial cell. In this respect, IS1 may be an intracellular parasite even more primitive than bacteriophages or bacterial plasmids.

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Non-parallel evolution of metabolic and growth-promoting functions of insulin

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Insulin has both metabolic and growth-promoting activities¹⁻³. From extensive studies on the relative potencies of naturally occurring and chemically modified insulins, a region of the insulin molecule that is essential for its metabolic effects has been proposed⁴. Here, we have compared the effects of 23 different insulins and insulin analogues on growth (thymidine incorporation into DNA) and metabolic activities (glucose oxidation). For many insulins striking differences in potency in these two assays were observed. Most exhibited greater relative activity in growth-promoting than in the metabolic assay, suggesting that growth and metabolic activities of the insulin molecule have evolved in a non-parallel fashion and may involve separate functional domains of the molecule.

Insulin effects at the cellular level are diverse. Exposure of cells to low concentrations of insulin results in a rapid stimulation of a variety of enzymes and transport systems involved in cellular metabolism⁵⁻⁷. At higher concentrations and after longer times, insulin also exerts growth-promoting effects with stimulation of macromolecular synthesis^{3,8,9}. Using antibodies to the insulin receptor, we have recently shown that the metabolic activity of insulin is mediated by the insulin receptor, whereas the growth-promoting effects are probably mediated through one of the receptors for the insulin-like growth factors (IGFs)¹⁰. The latter are a family of polypeptides that are structurally homologous to insulin and share many common biological activities, but are immunologically distinct⁹. The IGFs include IGF-I, IGF-II, somatomedins A and C and multiplication stimulating activity (MSA). In addition, IGFs have their own cell-surface receptors⁹⁻¹². Whereas the biologically active site of insulin for metabolic activity and insulin receptor binding has been extensively mapped^{4,13}, little information exists on the structural requirements for its growth-promoting activity. To this end we have compared the effects of a wide variety of insulins and IGFs in two bioassays.

Dose-response curves of insulins for stimulation of glucose oxidation in dispersed rat epididymal adipocytes demonstrated the wide range of potencies of insulin analogues for metabolic effects (Fig. 1a). For turkey, pork and bonito insulins, proinsulin and desoctapeptide insulin, the relative potencies (%) in this assay were 240, 100, 42, 2 and 0.4, respectively. In contrast, dose-response curves for the stimulation of thymidine incorporation into DNA of cultured human fibroblasts by these same analogues showed both different relative potencies and a

Table 1 Comparison of insulins and IGFs in metabolic and growth assays

Insulin	(ED ₅₀ pork/ED ₅₀ analogue) × 100		B/A
	Stimulation of glucose oxidation (A)	Stimulation of thymidine incorporation (B)	
Pork (monocomponent)	100	100	1.0
Beef	100	98	1.0
Human (synthetic)	94	120	1.3
Mouse	82	130	1.6
Sheep	75	100	1.3
Horse	87	112	1.3
Proinsulin (pork)	2	51	26
Proinsulin (beef)	3	63	21
Turkey	240	350	1.5
Bonito (fish)	42	160	3.8
Hagfish	7	8	1.1
Guinea pig*	3	12	4
Porcupine*	3	110	37
Coyu*	1	190	190
Casiragua*	2	43	22
Desoctapeptide (DOP) insulin	0.4	30	75
Des-Ala des-Asp insulin	5	10	2
Des-Gly A1 des-Phe B1 insulin	0.8	14	18
Butoxycarbonyl A1 insulin	50	80	1.6
A1-B29 adipoyl insulin	6	98	16
Insulin-like growth factor I (IGF-I)	0.2	860	4,300
Insulin-like growth factor II (IGF-II)	1.0	430	430
Multiplication stimulating activity (MSA)	0.2	120	600

Stimulation of glucose oxidation was measured in dispersed epididymal adipocytes of Sprague-Dawley rats (100–140 g) as the conversion of ¹⁴C-glucose to ¹⁴CO₂ as described by Rodbell *et al.*⁵. For measuring incorporation of ³H-thymidine into DNA of cultured human fibroblasts⁸, cells used were from the 5th to 15th passages. After each passage, the cells were allowed to reach confluency with media containing 20% fetal bovine serum (Flow Labs 29101420), then maintained in serum-free Eagle's minimal essential media for an additional 5–7 days before use. The results shown are the average of quadruplicate assays. A control dose-response curve with porcine insulin was done with each experiment. Inter- and intra-assay variations were less than 25% and 20%, respectively.

* ED₅₀ values for the hystricomorphs were derived by taking the concentration of insulin needed to stimulate thymidine incorporation that corresponds to the half-maximum of pork insulin's dose-response curve.

different order (Fig. 1b). Turkey (350%) and bonito insulins (160%) were more potent than pork insulin. Porcine proinsulin and desoctapeptide insulin remained less potent than pork insulin, but both were considerably more potent in the thymidine incorporation assay than in glucose oxidation. These differences in potency were not due to a change in the rate of degradation (data not shown). Furthermore, the differences are unlikely to be due to contamination with IGFs, because fully

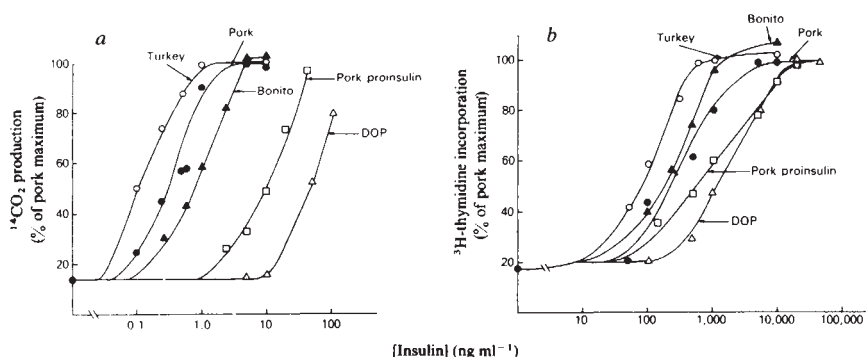


Fig. 1 Dose-response curves of some naturally occurring and chemically modified insulins for the stimulation of glucose oxidation in rat adipocytes (a) and thymidine incorporation into DNA in human fibroblasts (b). The methods are described in Table 1 legend.

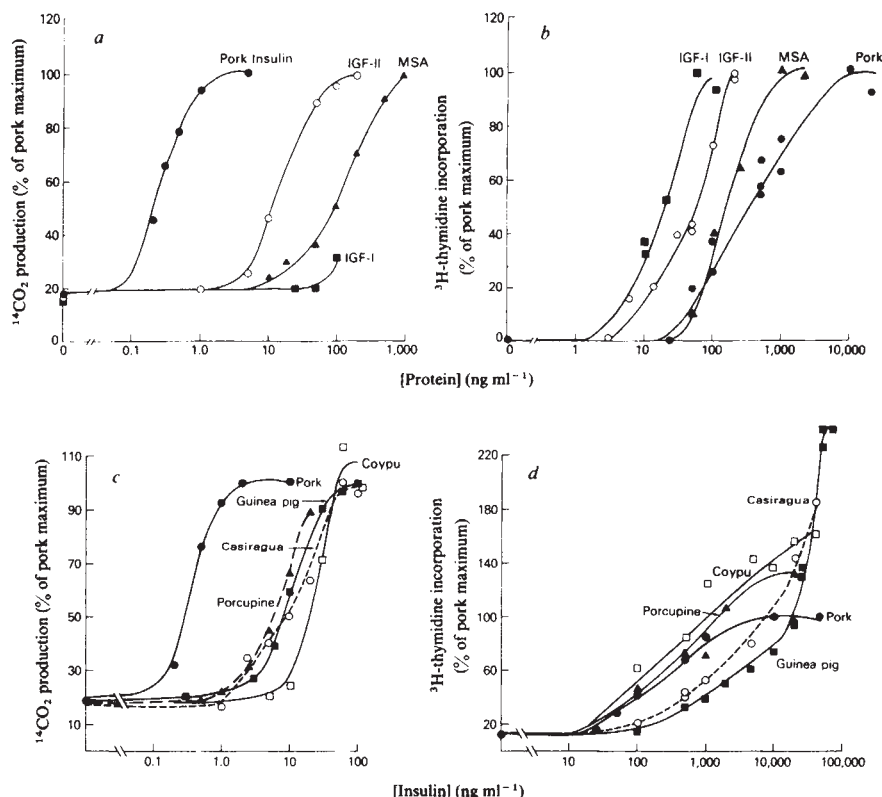


Fig. 2 *a, b*, Dose-response curves of pork insulin and various IGFs for the stimulation of glucose oxidation in rat adipocytes (*a*) and thymidine incorporation into DNA in human fibroblasts (*b*). *c, d*, Dose-response curves of pork insulin and various hystricomorph insulins for the stimulation of glucose oxidation in rat adipocytes (*c*) and thymidine incorporation into DNA in human fibroblasts (*d*). The methods are as described in Table 1 legend.

synthetic human insulin is equally potent with pork insulin in both bioassays (Table 1 and ref. 14).

The divergence between insulin's metabolic and growth effects is further substantiated by data with other insulin analogues (Table 1). With the exception of hagfish insulin, all the naturally occurring insulins were at least 50% as active as pork insulin in the thymidine incorporation assay, suggesting that insulin's growth effects have withstood evolutionary changes in the molecule better than its metabolic effects. This observation is strengthened by the results with chemically modified insulins which have retained their growth activity to a greater degree than their potencies in glucose oxidation.

The most extreme examples of divergence in the two assays are shown by IGF-I, IGF-II and MSA (Fig. 2*a, b*). All these growth factors have <1% of pork insulin's metabolic potential, with insulin > IGF-II > MSA > IGF-I. However, when comparing their growth potentials, the order of potencies is completely reversed, with IGF-I being the most potent and insulin the least.

At high concentrations almost all the insulins and IGFs produced the same maximal response in the bioassays. Furthermore, insulin's growth effect on the human fibroblast was not additive to that of the IGFs (ref. 11 and data not shown), suggesting that these peptides have a common rate limiting step either at the receptor or beyond.

However, insulins from the family of mammals referred to as hystricomorphs (guinea pig, porcupine, coypu and casiragua) did not conform to this finding. These insulins differ from other mammalian insulins in that they are unable to bind zinc, and exhibit low degrees of dimerization and low potency for metabolic effects^{4,15,16} (Fig. 2*c*). In contrast, when these insulins were studied in the thymidine incorporation assay, two points of interest emerged (Fig. 2*d*). First, like many other insulins, at low concentrations the hystricomorph insulins exhibited a greater relative potency in the growth assay than in their metabolic effects. More importantly, at higher concentrations these insulins stimulated thymidine incorporation into DNA to a greater maximum than any other insulin, insulin-like growth factors or combination of the two tested (Fig. 2*d*). In fact, at high concentrations guinea pig insulin approached the activity of

20% fetal calf serum, the most potent stimulus known in this assay.

From these data, some of the properties of the insulin molecule that are responsible for growth can be deduced. First, the growth-promoting action of insulin can tolerate a greater change in structure than the metabolic effects. Specific substitution of histidine in the A8 (A chain, residue 8) residue of insulin (for example, turkey insulin) enhances both its metabolic¹⁷ and growth effects. The B22-B30 region of insulin, which is vital for insulin's metabolic activities⁴, is not crucial for insulin growth effects. This is clearly demonstrated by the results of desoctapeptide insulin, and is consistent with the model for the structure of insulin-like growth factor I proposed by Blundell *et al.*¹⁸ in which this portion of the molecule is covered. Finally, the unexpected finding that the hystricomorph insulins gave a higher maximum response than other insulins in the growth-promoting assay raises the possibility that they are closer functionally and evolutionarily to the IGFs than other mammalian insulins. Four areas of similarities between the sequences of hystricomorph insulins and IGFs may be important: B10 (Asn, Gln and Glu in the hystricomorphs and IGF; His in other insulins), B13 (Asp in IGF and hystricomorphs; Glu in others), B26 (Tyr in coypu, casiragua and IGFs, Phe in others) and A4 (Asp in hystricomorphs, Glu in others).

The present data thus indicate that the growth-promoting activity of the insulin molecule is distinct from its metabolic activity and is less affected by amino acid substitutions. Presumably, this reflects differences in the specificity of the receptors for the IGFs and insulin. The insulins from the hystricomorph family seem to be unique in their ability to stimulate DNA synthesis. Preliminary studies in our laboratory suggest that this superagonist property can be explained by a receptor which uniquely recognizes the hystricomorph insulins, indicating that the hystricomorph insulins may represent another class of 'insulin-like growth factors'.

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butoxycarbonyl A1, and A1-B29 adipoyl insulins), R. E. Humbel (IGF-I and IGF-II), and M. M. Rechler and S. P. Nissley (MSA) for various insulins noted in Table 1. All other insulins were from Novo. We also thank Dr Jesse Roth for his guidance, Dr T. B. Blundell for suggestions in the project and Mrs Nancy Conley for secretarial assistance.

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Is the similarity of monozygotic twins due to genetic factors alone?

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It is generally assumed that the identity of the genotype explains why there is less difference between monozygotic than between dizygotic twins. But monozygotic twins are also exposed to identical non-genetic influences, which act on the common zygote until the time of separation into the two twin partners. We have studied such influences by comparing genetically identical monozygotic and dizygotic mouse twins transplanted into foster mothers at the 8-cell stage. We show that monozygotic twins have a greater degree of similarity than genetically identical dizygotic twins. This suggests that the differences between dizygotic twins may be due in part to non-genetic influences on the zygote as far as the third cleavage stage.

Differences between monozygotic (MZ) twins derived from a single zygote of an inbred line were compared with differences between dizygotic (DZ) twins from two ova of one mother of the same line. Body weight and onset of signs of somatic maturation (first appearance of hair, eye and ear opening) were determined. The high degree of isogenicity of inbred mice has been discussed elsewhere¹⁻⁴. Our experiments were carried out on inbred AKR/NHan mice derived from brother-sister mating over more than 150 generations. Isogenicity in these inbred strains was continuously verified by testing different immunogenic and biochemical genetic markers, and by measuring morphometrical traits of the mandibula. Over 100 gene loci have been tested by the breeding units at Zentralinstitut für Versuchstiere, FRG; the results have been reported elsewhere⁴.

MZ twins were produced as follows: ova were collected by flushing the tuba uteri on day 3½ post coitum (p.c.) (vaginal plug = day 1) after spontaneous ovulation. The flushing fluid⁵ contained phosphate-buffered saline (PBS) supplemented with 20% heat-inactivated (30 min at 56 °C) fetal calf serum (FCS). The zona pellucida was removed by incubation with 0.5% pronase⁶ and the denuded ova were cultured in the flushing medium at 37 °C for 3 h in an atmosphere of 95% air, 5% CO₂ (refs 7, 8). The 8-cell stages, in a microdrop of the flushing medium, were mechanically separated under liquid paraffin using an extended glass capillary tube, and both halves were incubated for 3 h as described above. The two halves, together with five to seven denuded 8-cell stages of C57BL/6JHan were then transferred to the uterus of 2½-day pseudopregnant, uniparous C57BL/6JHan recipients^{6,9}. DZ twins were produced in the same way except that, instead of two halves of one 8-cell-stage zygote, two undivided, denuded 8-cell-stage zygotes derived from one mother were transferred.

Animals were born at ~day 20 p.c. The litters of 4-8 offspring each were suckled by their uterine foster mothers, weaned at 25 days of age, and the males and females of each litter placed in separate cages. All animals were examined daily for health and signs of maturation, and weighed every 10 days from 31 to 101 days old. MZ and DZ AKR twins were identified by their white hair among their black C57BL/6JHan littermates. In ~200 transfer experiments, 10 litters had living MZ twins. In the same period, 13 pairs of DZ twins were produced in ~50 attempts. For each of the measured characteristics mean and standard deviation, and 'within pairs' (MS_w) and 'between pairs' (MS_b) mean squares were calculated. Analysis of variance model II (ref. 10) was used to calculate the component of variance (s_b²) for the variation between the pairs in each group of MZ and DZ, that of male and female twin pairs separately, using the formula: s_b² = 0.5 (MS_b - MS_w). A typical calculation is given in Table 1.

Table 1 Body weights of pairs of 51-day old male MZ and DZ and estimation of mean squares and components of variance analysis model II (random effects)

					Body weight (g)				
					MZ		DZ		
					23.0	21.5	28.0	21.5	
					23.0	25.5	27.0	26.5	
					30.0	31.0	26.0	24.0	
					29.0	27.0	21.0	25.0	
					27.0	27.0	24.5	22.5	
					26.0	25.5			
					28.0	28.0			
					26.5	24.5			
					26.2	± 2.66	24.6	± 2.37	
					Mean square	Components of variance (s ²)	Sum of squares	MS	Components of variance (s ²)
					d.f.		d.f.		
Total	15	99.6	6.64	—	9	—	50.4	5.60	—
Between	7	90.7	12.97	5.93	4	—	17.2	4.3	1.18
Within	8	8.9	1.11	1.11	5	—	33.3	6.65	6.65
					$F = 6.65/1.11 = 5.99$				
					$P < 0.005$				

Table 2 Mean $\pm$ s.d., MS_w and s_b^2 estimated for age of MZ and DZ at first appearance of hair, and eye and ear opening of males and females combined, and for body weight of males and females of different age

Character	Mean $\pm$ s.d.		Component of variance			
	MZ	DZ	MZ	DZ	F	P
Appearance of hair (days)	6.00 $\pm$ 1.65 (20)	6.35 $\pm$ 1.32 (26)	s_b^2 2.89 MS_w <0.001	0.91 0.88	3.18 880.00	<0.05 <0.001
Eye opening (days)	14.20 $\pm$ 2.37 (20)	13.65 $\pm$ 2.65 (26)	s_b^2 5.96 MS_w <0.001	6.17 1.11	1.04 1,110.00	NS <0.001
Ear opening (days)	13.60 $\pm$ 3.01 (20)	13.50 $\pm$ 2.86 (26)	s_b^2 9.60 MS_w <0.001	7.22 1.20	1.33 1,270.00	NS <0.001
Body weight (g), δ day 31	17.50 $\pm$ 3.53 (16)	15.80 $\pm$ 2.79 (10)	s_b^2 11.19 MS_w 2.06	4.15 4.10	2.70 1.99	NS NS
Body weight (g), ϕ day 31	13.50 $\pm$ 3.46 (4)	15.41 $\pm$ 3.35 (16)	s_b^2 18.00 MS_w <0.00005	3.12 8.33	5.77 13,900.00	NS <0.001
Body weight (g), δ day 41	23.34 $\pm$ 3.01 (16)	20.70 $\pm$ 2.20 * (10)	s_b^2 9.05 MS_w 0.61	1.80 3.25	7.67 5.33	<0.05 <0.05
Body weight (g), ϕ day 41	20.75 $\pm$ 1.44 (4)	19.50 $\pm$ 3.54 (16)	s_b^2 3.12 MS_w <0.00005	3.32 9.47	1.06 189,400.00	NS <0.001
Body weight (g), δ day 51	26.41 $\pm$ 2.58 (16)	24.60 $\pm$ 2.37 (10)	s_b^2 5.93 MS_w 1.11	5.93 6.65	5.03 5.99	NS <0.05
Body weight (g), ϕ day 51	23.87 $\pm$ 1.31 (4)	22.56 $\pm$ 3.81 (16)	s_b^2 2.53 MS_w 0.06	6.06 8.87	2.40 147.83	NS <0.01
Body weight (g), δ day 61	28.78 $\pm$ 2.27 (16)	27.20 $\pm$ 2.29 (10)	s_b^2 5.21 MS_w 0.26	2.49 7.50	2.09 28.85	NS <0.01
Body weight (g), ϕ day 61	25.5 $\pm$ 0.91 (4)	26.22 $\pm$ 4.12 (16)	s_b^2 1.06 MS_w 0.12	3.83 13.39	3.61 111.06	NS <0.01
Body weight (g), δ day 71	30.34 $\pm$ 2.64 (16)	28.95 $\pm$ 2.69 (10)	s_b^2 7.07 MS_w 0.39	1.94 5.52	3.64 14.15	NS <0.01
Body weight (g), ϕ days 81, 91, 101	32.60 $\pm$ 2.94 (16)	29.68 $\pm$ 4.11 (10)	s_b^2 7.70 MS_w 1.13	7.64 9.49	1.01 8.40	NS <0.01

Mean $\pm$ s.d., MS_w and the between pairs component of variance (s_b^2) estimated in groups of MZ and DZ twins for the day of first appearance of hair, and eye and ear opening of male and females combined, and for the body weights (g) of males and females of different age. Numbers in parentheses are number of animals. NS, not significant.

* Means are significantly different ($P < 0.05$).

The F -test was used to compare s_b^2 and MS_w between the MZ and DZ twin groups; the results are shown in Table 2. The mean age at first appearance of hair and of eye and ear openings did not differ ($P > 0.5$) between MZ and DZ groups. In addition, the mean body weights of MZ and DZ did not differ ($P > 0.1$) in 15 out of 16 measurements made between 31 and 101 days old. The mice stopped growing at ~ 70 days old; the mean body weight at 81, 91 and 101 days remained constant, therefore the results of these ages were combined.

A comparison of the total variance between MZ and DZ with respect to the measured characteristics reveals no detectable trend. MS_w and s_b^2 for the pairs of twins are significantly different in the two groups (see Table 2, columns 4–8). In monozygotic twins MS_w is extremely small, most of the variance being due to s_b^2 . In dizygotic twins MS_w was greater than s_b^2 for 9 of the 13 measurements. MS_w for the monozygotic twins was significantly smaller than that of the dizygotic twins, for the observed ages of maturation and body weights in males and females up to the time of maturation (61 days). This difference remained significant in males even after maturation, but it disappeared in adult females. From repeated weight estimation for each MZ and DZ adult male at 81, 91 and 101 days of age the intra-individual component of variance was calculated to be 2.39^2 g. This component of variance is approximately equal to the MS_w of the MZ but is smaller ($P < 0.05$) than that in DZ.

Thus in spite of the fact that both MZ and DZ were isogenic and developed in identical pre- and postnatal environments, the MZ pairs showed fewer differences than the DZ pairs. A possible explanation for these findings is residual heterozygosity in the AKR inbred strain, but there is no support for such a hypothesis. We are following a divergent breeding programme in our AKR/NHan strain that is selective for the characteristics under study, to provide further evidence for the homogeneity of the gene loci involved. An alternative explanation for the larger differences observed in the DZ twins is that experimental manipulation differed between the MZ and DZ groups; whereas the 8-cell embryos of the MZ group were divided, those of the

DZ group were not. However, it is difficult to see how experimental manipulation of this kind could create greater similarity. To investigate this further, control studies are being done in which, for example, two embryos at the 8-cell stage are divided and one 4-cell entity from each is transferred to a common foster mother.

From the available evidence, we conclude that each zygote is modified by a non-genetic mechanism before the third cell division in such a way that postnatal maturation times and body weights and possibly other characteristics are considerably different between individuals. If a zygote is divided after the third cell division, the modification is decisive for both monozygotic partners in the same way. It has long been known that there is a non-genetical component of variance of many quantitative characteristics which could not be accounted for by environmental influence, that is, by circumstances external to the individual. This component was called 'intangible variance'^{11,12} and was attributed also to 'accidents' of development. We believe that the quantitative component of variance described here is a manifestation of such intangible variance.

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Population genetics of selfish DNA

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Selfish DNA, which contains no genetic information but which is perpetuated in eukaryote genomes, has attracted much attention recently¹⁻⁴. Two classes of selfish DNA have been distinguished: tandemly repetitive sequences such as satellite DNA and dispersed repeated families. Unequal crossing-over is considered to be mainly responsible for spreading of the former, and an integration mechanism (as in bacterial transposons) for evolution of the latter. Here I derive equations and simulate distributions (using randomly generated numbers as data) to show how the numbers of tandem and dispersed repeat units of selfish DNA in a population change with time. I find that whereas the mean number of clustered and dispersed units remains constant, the distribution becomes increasingly dispersed with time.

Both unequal crossing-over and integration occur randomly, such that the overall mean change of unit number is zero. I assume that the number of units per genome in an evolving population changes by inter-genome exchange as well as by random sampling drift at reproduction. How does the mean number of units per genome of the population drift with time? In the previous study⁵, the relationship between the mean and variance of the number of units in the population was obtained when the above processes balance each other. I shall start from such an equilibrium situation. The basic premise of this study is that self-replication has a more profound effect on the evolution of selfish DNA than individual base substitution, on which evolution of ordinary stable gene loci depends.

The same model as in ref. 5 is used; however, the tandem and the dispersed repeated sequences are here treated separately. Each repeating unit of the dispersed class is assumed to have a constant probability α_1 of either forming an additional copy of itself or being deleted in one generation. Exchange of units between the genomes also takes place in each generation at rate β . At meiosis, the units are assumed to be randomly distributed to the two daughter cells by the normal methods of chromosomal segregation. Both unequal sister-chromatid exchange (rate per generation α_2) and unequal inter-chromosomal crossing-over change the amount of clustered selfish DNA. It is assumed that the mean number of duplicated or deleted units in one generation is proportional to n , the number of repeating units in a genome (constant of proportionality, a). Inter-chromosomal crossing-over between the homologous repeated DNA is complicated because the two genomes usually have different numbers of repeating units. Recombination can be either 'equal' or 'skewed' depending on where the smaller segment of repeated DNA comes in relation to the larger segment. Equal recombination occurs when the small segment falls completely within the larger segment, and skewed recombination when it does not. The equal recombination rate per generation is denoted by β_1 and the skewed one, by β'_1 . In addition to the above processes, random sampling of gametes occurs at reproduction (number of breeding individuals, N). The parameters of various processes for the two classes are summarized in Table 1.

Let x and y be the average number of units per genome in the populations of tandem and the dispersed repeated sequences respectively. They are random variables and, in the following, their distribution is investigated as function of time. In numerical experiments I generate data starting off with random numbers, and study the distribution obtained using the equations derived below.

The 'pseudosampling (PSV)' method of Kimura⁶ is used, which greatly facilitates the simulation. If x is the average number of units in the present population, then its number in the next generation, x' , is given by

$$x' = x + \xi_{\text{psv}} \quad (1)$$

where ξ_{psv} is a uniform random number with mean 0 and the variance expected after the occurrence of the various processes given in Table 1. The variance ($V_{\Delta x}$) of change of x (ξ_{psv}) may be calculated using equations (5) and (12)–(14) of ref. 5, and by noting that x is the average of $2N$ variables. Note further that each genome undergoes, independently of the other, replication and exchange processes, before it is sampled for the next generation. Also, equation (13) of ref. 5 is equivalent to stating that the square of the average number of units increases by the absolute value of the right term of this equation by sampling of gametes. It becomes

$$V_{\Delta x} = \frac{x^2}{2N} \left(\alpha_2 a^2 + \frac{\beta'_1}{6} \right) \left\{ 1 + \frac{6(\alpha_2 a^2 + 1) - 2\beta_1 - \beta'_1}{2\beta_1 + \beta'_1 + 3N^{-1} - 6\alpha_2 a^2} \right\} \quad (2)$$

by assuming that $2\beta_1 + \beta'_1 + 3N^{-1} > 6\alpha_2 a^2$. The simulation is continued, starting from a given value of x at time 0, for 50N generations, and it is repeated 1,000 times. Whenever x becomes $< 1/N$, x is put to 0, and the population has lost the

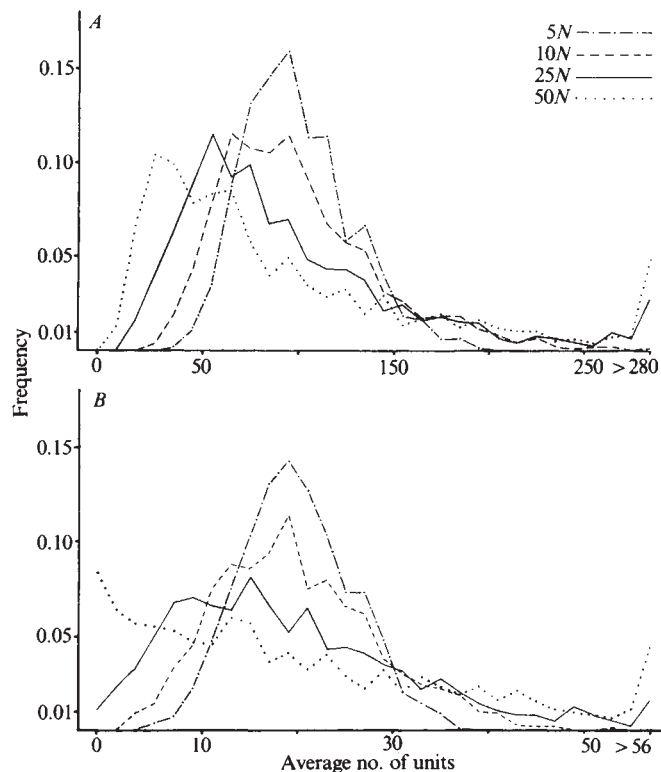


Fig. 1 A, results of simulations of the distribution of the average number of tandemly repeated units per genome in the population. Parameters are: initial number of units (x_0) = 10^2 ; unequal cross-over rate (α_2) = 10^{-2} ; fraction of units duplicated or deleted (a) = 0.1; equal recombination rate (β_1) = 10^{-2} ; skewed recombination rate (β'_1) = 10^{-3} ; and $N = 10^4$. Distribution was obtained for the intervals of 10 units, with terminal classes of 0 and > 280 units. B, results of simulations for the dispersed repeating sequences. Parameters are: initial number of units (y_0) = 20; rate of duplication or deletion of each unit (α_1) = 10^{-2} ; rate of inter-genome exchange (β) = 1; and $N = 10^2$. The product, $N\alpha_1$, is chosen to be reasonably realistic. Distributions are for the intervals of 2 units with terminal classes of 0 units and > 56 units. The distributions for $t = 5N, 10N, 25N$ and $50N$ generations are given. They are the result of 1,000 independent trials.

Table 1 Processes responsible for the evolution of selfish DNA and the parameters used

Processes	Tandemly repeated sequences (x)	Dispersed repeated sequences (y)
Replication process	Unequal crossing-over by sister-chromatid exchange α_2 = rate per cluster per generation a = mean fraction of duplicated or deleted units at an unequal crossing-over in one cluster of repeated sequence	Integration mechanism α_1 = rate in one generation at which each unit is either duplicated or deleted independently
Inter-genome exchange process	Inter-chromosomal recombination at meiosis β_1 = rate of recombination in one generation which takes place in this sequence when the pairing of two chromosomes is equal β_1 = the above rate when the pairing of two chromosomes is skewed	Random distribution to the daughter cells at meiosis β = rate per generation at which the dispersed units are distributed
Random genetic drift	N = effective population size	N = effective population size

repeating sequence. In practice, ξ_{psv} may be obtained by choosing a random number rnd (uniformly distributed between 0 and 1) such that

$$\xi_{psv} = \sqrt{3V_{\Delta x}} (2rnd - 1) \quad (3)$$

(See ref. 6.)

The distribution of y (the mean number of units of the dispersed family) is similarly studied by using $y' = y + \xi_{psv}$. ξ_{psv} now follows a uniform distribution with mean 0 and variance that equals the variance of the change of y after the occurrence of the three processes of Table 1. The variance takes the following form from equations (3), (8), (13) and (14) of ref. 5:

$$V_{\Delta y} = \frac{y}{2N} \left(\alpha_1 + \frac{N\alpha_1\beta}{1+2N\alpha_1} \right) \left(1 + \frac{2-\beta}{\beta+N^{-1}} \right) \quad (4)$$

From equations (2) and (4), it is possible to approximate the variance of x or y at any time, that is, the variance of the number of units among the isolated populations. They may be expressed as

$$V_{x,t} = V_{\Delta x_0} t \quad (5)$$

and

$$V_{y,t} = V_{\Delta y_0} t \quad (6)$$

where t denotes the t th generation after splitting of the populations, and $V_{\Delta x_0}$ and $V_{\Delta y_0}$ are obtained by replacing x of equation (2) with x_0 , and y of equation (4) with y_0 respectively, where x_0 and y_0 are the numbers of units of clustered and dispersed classes at $t = 0$. As β (in equation (4)) is likely to be unity in sexually reproducing species (because the rate at which the dispersed family is divided into the daughter cells at meiosis is unity), the product $N\alpha_1$ is the most crucial factor in determining the distribution of dispersed families.

Figure 1A,B shows the results of simulation. The values of the parameters used are not known exactly in real cases and are tentative suggestions. Note that several dispersed families are known in which the number of units per genome is of the order

of 10 (refs 7–10) and that the parameters α_1 and N are chosen such that the product, $N\alpha_1$, is a realistic value ($= 1$). For the clustered families, reasonable values are also chosen^{11–14}, except for N , which is too small but makes the simulation easier because of more rapid differentiation. The figure shows, starting from the initial number of units, how the distribution of x or y changes with time. For example, after $10N$ generations, the value of x ranges from 30 to 290 with the peak at about $x_0 = 100$, and y , from 3 to 53, with the peak at $y_0 = 20$. As t increases, the distribution becomes more and more dispersed in both classes. Nevertheless, the overall mean of 1,000 replicated lines stays constant; at the $50N$ th generation, $\bar{x} = 98.1$ and $\bar{y} = 19.9$. Such an outcome would be characteristic of random processes of this kind. Note that, at this generation, $\sim 8\%$ of 1,000 lines have lost the dispersed repeating sequence, and about the same proportion contained less than 20 copies of the clustered class. Such a proportion would increase with time even if the overall mean remained unchanged.

The variances of x and y have also been calculated and compared with the theoretical predictions from equations (5) and (6), as shown in Table 2. The agreement between the theoretical and simulated results is generally satisfactory; however, for the clustered families, equation (5) underestimates the true value when t gets large. This is because the number of units duplicating or being deleted is assumed to be proportional to the number of units, and therefore the variance of the change of x is proportional to x^2 , the expectation of which becomes larger than x_0^2 as t gets large.

It has been shown that whereas some species have the same α satellite DNA¹⁵, other closely related species have lost the sequence^{16,17}. It has thus been argued that this sequence may be important in speciation¹⁶. However, as can be seen from the present results, spreading and loss of repeated sequences occur randomly and it is extremely difficult to draw any conclusion from such an observation. Data on species comparisons of dispersed repeated sequences are also rapidly accumulating (see ref. 7 for review). Most examples seem to fit in with the framework of the present theory.

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Table 2 Comparison of the observed and expected variances of the average number of units per genome among the isolated populations

Time in generations ($\times N$)	Tandemly repeated		Dispersed repeated	
	Observed variance	Expected variance	Observed variance	Expected variance
5	768	798	32.9	34.2
10	1,717	1,595	69.4	68.3
15	2,480	2,393	97.9	102.5
20	3,816	3,190	137.5	136.7
25	4,672	3,988	166.3	170.8
30	6,003	4,785	200.9	205.0
35	7,112	5,583	237.9	239.1
40	8,568	6,381	274.8	273.3
45	8,852	7,178	300.3	307.5
50	9,484	7,976	320.8	341.6

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Inhibition of actin polymerization in blood platelets by cytochalasins

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It has been suggested that the polymerization of actin to form actin filaments is critical for many functions in non-muscle cells including phagocytosis, cell division, secretion and cell motility^{1,2}. In support of this suggestion, many of these functions are inhibited by cytochalasins³ which also inhibit the actin nuclei-induced polymerization of purified actin⁴⁻⁷. Recent studies suggest that the cytochalasins (cytochalasins B, D and E) inhibit polymerization by binding to the growing end of actin filaments and blocking the further addition of monomeric actin molecules^{4,7-9}. If the cytochalasins inhibit cellular contractile functions by a similar mechanism, they could be useful tools in determining whether actin polymerization is required for specific processes within cells. Blood platelets provide an excellent system for testing this as exposure of platelets to thrombin results in a rapid polymerization of actin^{10,11}. We have now studied the effects of the cytochalasins on thrombin-induced actin polymerization and report here the first clear evidence that cytochalasins inhibit actin polymerization in intact cells.

The filamentous actin content of platelet samples was determined in platelet lysates by the method of Blikstad *et al.*¹² who showed that nonfilamentous actin inhibits DNase I activity while filamentous actin has little effect unless depolymerized by guanidine hydrochloride. Platelets were lysed by adding an equal volume of Triton extraction buffer (2% Triton X-100 (v/v), 10 mM EDTA, 100 mM Tris-HCl, pH 7.4). This buffer, unlike that used elsewhere^{10,12}, did not depolymerize actin filaments, so that identical inhibitory values were obtained whether the assay was performed 30 s or 2 h after lysis (J.E.B.F., M. E. Dockter & D.R.P., in preparation).

In Triton lysates from unstimulated platelets, the DNase I inhibitory activity in the absence of guanidine hydrochloride was ~50% of that in its presence. When platelets were activated with thrombin before the addition of Triton X-100, the amount of guanidine hydrochloride-dependent DNase I inhibitory activity increased to ~70% of the total. This stimulus-induced transformation was rapid and was almost complete within 15 s (Fig. 1). We conclude that ~50% of the total actin was in a filamentous form in unstimulated platelets and that this increased to ~70% on thrombin activation. In support of this conclusion, we found that only the guanidine hydrochloride-dependent inhibitory activity was sedimented by centrifugation of lysates at 100,000 g for 3 h. Furthermore, sedimented material contained structures morphologically similar to actin filaments¹³. Finally, quantitation of actin in sediments and supernatants by densitometry of Coomassie blue-stained gels after SDS-polyacrylamide gel electrophoresis gave actin contents that were within 5% of those obtained by the DNase I inhibition assay. Thus, the amount of inhibition of DNase I activity in Triton lysates correlates directly with the amount of nonfilamentous actin (an assumption used throughout this study), and the amount of filamentous actin within platelets rapidly increases after exposure to thrombin.

Figure 1 shows that 10^{-6} M cytochalasin E added 100 min before thrombin inhibited the thrombin-induced increase in actin filament content. Cytochalasins D and B had similar effects. None of these cytochalasins had any effect when added

simultaneously with the Triton extraction buffer. As shown in Table 1, the inhibitory effect was rapid, as the same extent of inhibition was observed whether cytochalasin D was present for 100 min or for only 1 min before the addition of thrombin. Cytochalasin D did not affect the filament content in unstimulated platelets (Table 1). These data show that the decreased filament content in thrombin-treated platelets is due to an inhibition of thrombin-induced actin polymerization and not to

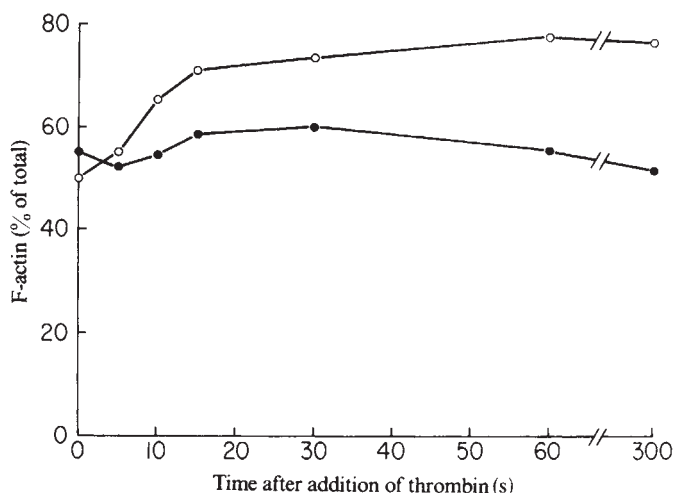


Fig. 1 Inhibition of thrombin-induced actin polymerization in human platelets by cytochalasin E. Human platelets from freshly drawn blood were washed as previously described²⁴ and suspended to a concentration of 2×10^8 per ml in 138 mM sodium chloride, 2.9 mM potassium chloride, 12 mM sodium bicarbonate, 0.36 mM sodium phosphate, 5.5 mM glucose and 1 mM EDTA, pH 7.4. The platelet suspension was incubated with 0.2% (v/v) DMSO (dimethyl sulphoxide) (O) or 10^{-6} M cytochalasin E in 0.2% DMSO (●) at 37 °C. After 100 min, thrombin was added (final concentration $0.1 \text{ NIH U ml}^{-1}$) and at intervals varying from 0 to 300 s, platelets were lysed by addition of an equal volume of Triton extraction buffer and a $10\text{-}\mu\text{l}$ aliquot removed for determination of the amount of unpolymerized actin by the DNase I inhibition assay. The total actin content in lysates was measured after depolymerization of filamentous actin by addition of an equal volume of buffer containing 1.5 M guanidine-HCl, 1.0 M sodium acetate, 1.0 mM sodium ATP, 20 mM Tris-HCl and 6 mM calcium chloride, pH 8.6 (to yield a final pH of 7.4, determined experimentally). Kinetic analyses were initiated within 30–60 s after platelet lysis. The absorbance was continually monitored by a dual-beam spectrophotometer directly linked to a Hewlett Packard 9845S computer. The linear portion of the sigmoidal curve was determined by regression analysis and runs were discarded if the correlation coefficient was less than 0.999. Duplicate determinations of the DNase I inhibitory activity from an individual platelet sample usually agreed within 5%. The actin filament content (F-actin) is expressed as a percentage of the total actin.

a decreased filament content of unstimulated platelets. Similar increases in actin polymerization were observed when platelets were stimulated for 30 s with $0.4 \mu\text{M}$ ionophore A23187 or for 60 s with $20 \mu\text{M}$ ADP. Cytochalasin D also inhibited these stimuli-induced increases.

Although the cytochalasins may affect many metabolic processes within cells, two sites of action that could potentially affect actin polymerization have been described: one site is on the actin filaments⁴⁻⁹; the other is on the membrane-bound glucose transport system^{14,15}. However these sites have differing affinities for the cytochalasins. The site on actin filaments has been measured using both purified actin^{4,8,9} and actin-containing membrane complexes^{5,16}. In both cases, cytochalasins D and E bound with higher affinity than cytochalasin B with the binding

affinity corresponding to the inhibition of actin nuclei-induced polymerization. In contrast, the site of the membrane-bound glucose transport system has a higher affinity for cytochalasin B than for cytochalasin D or E which, unlike cytochalasin B, do not inhibit glucose transport into cells¹⁴⁻¹⁸.

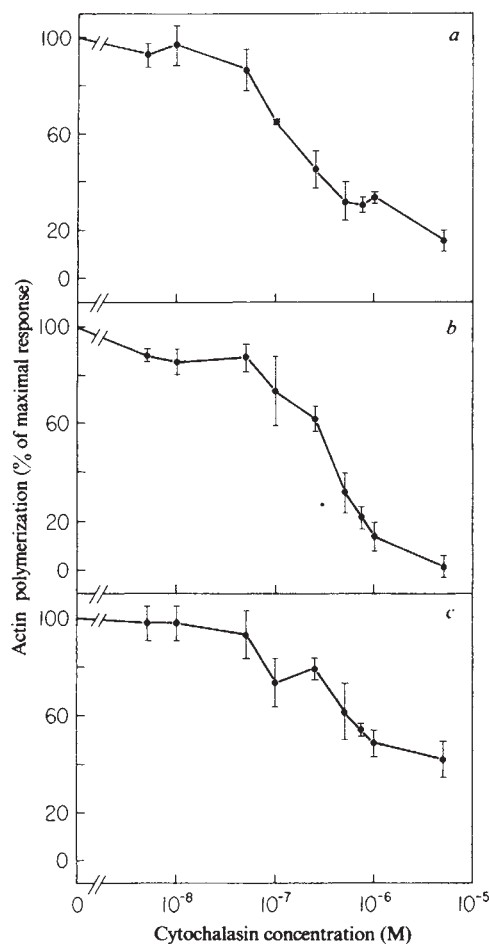


Fig. 2 Comparison of the effects of cytochalasins D(a), E(b) and B(c) on thrombin-induced actin polymerization in human platelets. Platelet suspension (2×10^8 per ml^{-1}) was incubated at 37°C with 0.5% ethanol (v/v) or with varying concentrations of the different cytochalasins in 0.5% ethanol. After 88 min, buffer or thrombin (final concentration $0.1 \text{ NIH U ml}^{-1}$) was added and 30 s later the platelets were lysed by addition of Triton extraction buffer. The actin filament content of lysates was determined as described in Fig. 1 legend and the thrombin-induced increase in actin filaments expressed as a percentage of the increase obtained when thrombin was added to platelets that had not been exposed to cytochalasin. Values shown are the mean $\pm$ s.e.m. from four donors.

Figure 2 compares the effects of the three cytochalasins on thrombin-induced actin polymerization. At $5 \times 10^{-6} \text{ M}$, cytochalasins D and E decreased the thrombin-induced actin polymerization by $\sim 85\%$ and 100% , respectively, while cytochalasin B inhibited polymerization by only $\sim 60\%$. Within the limits of experimental error, the concentrations of cytochalasins D and E required to produce half-maximal inhibition of polymerization were indistinguishable ($\sim 2 \times 10^{-7} \text{ M}$) and were lower than the concentration of cytochalasin B required for a similar inhibition ($\sim 10^{-6} \text{ M}$). These concentrations are similar to those that bind to actin filaments and inhibit actin polymerization in purified systems^{4,7,9}. The relative order of potency

Table 1 Effect of time of preincubation of platelets with cytochalasin D on the actin filament content of unstimulated and thrombin-activated platelets

Addition during preincubation	Stimulatory agent	Filamentous actin (% of total)					
		Time of preincubation (min)	1	5	20	70	100
Ethanol	None		52.1	51.6	48.1	48.2	50.5
	Thrombin		69.5	65.2	60.6	66.2	67.3
Cytochalasin D in ethanol	None		49.7	49.1	47.2	48.1	50.1
	Thrombin		54.4	56.1	51.6	55.4	56.3

Platelet suspensions were incubated with 0.5% ethanol (v/v) or with $2.5 \times 10^{-5} \text{ M}$ cytochalasin D in 0.5% ethanol at 37°C . At intervals from 1 to 100 min, buffer or thrombin was added and after 30 s, platelets were lysed with Triton extraction buffer and the actin filament content measured as described in Fig. 1 legend. Values given are the actin filament content as a percentage of the total platelet actin.

agrees with the notion that the cytochalasins inhibit actin polymerization by binding to preformed actin filaments and is inconsistent with a mechanism involving inhibition of glucose transport. In support of this view, the inhibitory effect of cytochalasin was rapid (Table 1), a result not expected if the drug inhibited glucose transport sites alone. In addition, concentrations of cytochalasin that completely inhibited thrombin-induced actin polymerization had no effect on the thrombin-induced phosphorylation of two platelet polypeptides¹⁹ or on the thrombin-induced association of myosin with platelet cytoskeletons¹³.

It has been proposed that actin filaments 'treadmill' where one end is a net polymerizing end and the other a net depolymerizing end^{20,25}. Experiments showing that the cytochalasins decrease the lengths of preformed actin filaments, unless the net depolymerizing ends are blocked, have been interpreted as evidence for such a mechanism⁸. If the cytochalasins inhibit thrombin-induced actin polymerization by binding to actin filaments, they must have access to the filaments before stimulation. Although the permeability of platelet plasma membranes to the cytochalasins has not been verified experimentally, we predict from their structure that they would readily cross plasma membranes. However, our results show that the cytochalasins had no measurable effect on the filament content when incubated for up to 100 min with unstimulated platelets (Table 1). This suggests that if 'treadmilling' occurs in unstimulated platelets, it occurs too slowly to be detected in the times used here, possibly because the depolymerizing ends are blocked.

Recently, Morris and Tannenbaum²¹ reported that cytochalasin D did not decrease the filament content of HEp-2 cells. These authors conclude that as cytochalasin D inhibits motile functions without decreasing filament content, the inhibitory effect is not due to a limitation of the availability of filaments. However, it is becoming increasingly clear that actin polymerization can occur on cellular stimulation^{10,11,22,23}. Such responses need not result in a net measurable increase in filament content as occurs within platelets, but may involve small increases in filament content within specialized areas of cells. Our observations that the cytochalasins inhibit actin polymerization in platelets suggest that cytochalasins may inhibit cellular processes³ by preventing the increased formation of actin filaments required for these processes. Experiments are now being done to determine whether the concentrations of cytochalasins that inhibit actin polymerization in platelets cause corresponding inhibition of platelet responses (shape change, aggregation, release of granule contents or clot retraction), and it is anticipated that similar studies will help to determine the role of stimuli-induced actin polymerization in other non-muscle cells.

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E. coli contains eight soluble proteolytic activities, one being ATP dependent

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Escherichia coli selectively degrades polypeptides with highly abnormal conformations^{1–4}. Normal cell proteins are degraded more slowly, but this process increases when the bacteria lack required nutrients^{1,5–7}. The pathways by which aberrant or normal proteins are hydrolysed are not known^{1,3,4}, but these processes require metabolic energy^{3–5,8,9}. Recently, cell-free proteolytic systems have been obtained from *E. coli*^{4,10} and mammalian cells^{3,11–15} that are stimulated by ATP. To clarify the pathway of intracellular protein degradation and to determine whether ATP might directly influence specific proteolytic enzymes, we have now undertaken to define the various proteases in *E. coli*. Our studies demonstrate the existence of eight distinct soluble proteolytic activities in *E. coli* extracts. Seven seem to be new enzymes. Six are serine proteases that hydrolyse ¹⁴C-globin and ³H-casein, while two others are metalloenzymes that degrade ¹²⁵I-insulin but not these larger polypeptides. One of the globin-degrading enzymes is stimulated markedly by ATP and thus may be the rate-limiting enzyme for protein breakdown *in vivo*.

Although several reports have concerned the isolation of proteases from *E. coli*, this field is in a confusing state. The so-called 'protease I' and 'protease II', which cleave chromogenic substrates for chymotrypsin and trypsin respectively^{16,17}, show little or no hydrolytic activity against proteins^{18,19}.

Furthermore, mutants lacking them have a normal capacity to degrade cell proteins^{19–22}. Another 'protease' has been purified²³ using a chromogenic substrate of subtilisin, but its ability to hydrolyse proteins has not been established. In addition, an aminopeptidase (pepN) has been purified, which has been claimed to have some endoprotease activity²⁴. An activity that can degrade casein, named 'protease A', has been partially purified²⁵ but it is probably not a single enzyme (see below). Cheng and Zipser²⁶ have purified an enzyme, protease III, that hydrolyses the amino-terminal fragments of β -galactosidase ('auto- α '), but it is not essential for protein degradation *in vivo*²⁷. Finally, these bacteria contain various peptidases that are essential for growth on peptides and hydrolyse small peptides generated during intracellular protein breakdown^{28,29}.

We therefore undertook a systematic search for protease activities in *E. coli*. For substrates, we used ¹⁴C-methyl-apohaemoglobin, ³H-methyl- α -casein and ¹²⁵I-insulin. Because insulin is a small polypeptide, it may be degraded by enzymes involved only in the later steps in the complete degradation of cell proteins^{29,30}. Extracts of *E. coli* K-12 hydrolysed all three substrates, but only degradation of globin was significantly stimulated by ATP. The degree of stimulation by ATP (60%) in these extracts, which were prepared with the French press from frozen cells, is smaller than the two- to threefold effects reported previously with fresh cells lysed by gentler methods^{10,30}.

To isolate the proteolytic activities responsible, we fractionated these extracts on DEAE-cellulose and eluted the adsorbed proteins with a NaCl gradient (Fig. 1). Five different peaks with proteolytic activity were found (designated I–V, according to their order of elution). These same peaks, and no additional ones, were found in several other *E. coli* K-12 strains that had been freshly grown and collected at either late log or stationary phase. Of special interest was the finding that one of the peaks that degraded globin and casein (IV) was stimulated dramatically by ATP, and this is presumably the activity responsible for the stimulation of globin and casein hydrolysis by ATP in the crude extracts¹⁰.

Peak I shows strong hydrolytic activity against globin and casein, but very slight activity against insulin. Peak I was further characterized by chromatography on CM-cellulose (Fig. 2) and by gel filtration on Sepharose 6B (data not shown). Both techniques resolved two distinct activities, both of which hydrolyse globin and casein. Peak IA shows on gel filtration an unusually large molecular weight (~500,000), whereas IB has a molecular weight of ~82,000. Both IA and IB are sensitive to diisopropyl fluorophosphate (DFP), and thus are serine proteases.

Peak II hydrolyses insulin, globin and casein. Isoelectric focusing (Fig. 3) and CM-cellulose chromatography at pH 5.2 (data not shown) can both resolve this fraction into three proteolytic peaks, one active against insulin and the other two against globin and casein. Both globin-degrading activities, IIB and IIC, seem to be serine proteases, but they differ in their relative sensitivities to DFP as well as in their heat stabilities³¹. The insulin-degrading enzyme (IIA) resembles in many respects protease III purified by Cheng and Zipser²⁶. Like protease III, peak IIA rapidly hydrolyses amino-terminal fragments of β -galactosidase (auto- α) and is sensitive to metal-chelating agents (EDTA and *o*-phenanthroline). Furthermore, a mutant lacking protease III (*ptr*), isolated by Cheng *et al.*²⁷, specifically lacked the insulin-degrading activity in peak II. These findings (K.H.S.S., C. H. Cheng and A.L.G., unpublished results) indicate that the insulin-degrading activity is identical to protease II.

Peak III hydrolyses globin (Fig. 1) and casein but not insulin. Because it is sensitive to DFP, the enzyme also seems to be a serine protease.

Peak IV was the one ATP-stimulated activity. The stimulation of globin or casein degradation by ATP ranged from 4- to 10-fold in the various fractions and required Mg²⁺ ions. The low amount of globin hydrolysis evident in the absence of ATP decreased further on use of a narrower salt gradient or DEAE-Sepharose, which resulted in an enzyme that seemed to be

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completely dependent on ATP. This enzyme showed no activity against insulin, which explains the finding that insulin hydrolysis was not stimulated by ATP in crude extracts. The ATP-stimulated activity shows the DFP-sensitivity characteristic of a serine protease.

Peak V contained insulin-degrading activity that was sensitive to *o*-phenanthroline but not to DFP. This peak did not digest

casein or globin. No additional proteolytic activity could be eluted with higher concentrations of NaCl.

These studies and related ones demonstrate the existence of eight soluble proteolytic activities in *E. coli*, all of which have now been purified to near homogeneity. The complete purification and characterization of these enzymes will be reported elsewhere (refs 31–33 and K.H.S.S., C. H. Chung and A.L.G., manuscripts in preparation). Of these, the six globin-degrading enzymes seem to be serine proteases. By contrast, the enzymes that degrade insulin (IIA and V) and similarly sized polypeptides are metalloproteases (Table 1). All these activities represent new enzymes³¹ except for the insulin-degrading enzyme (IIA), which seems to be identical to protease III²⁶. All these activities are also found in mutant strains lacking proteases I and II and aminopeptidase N. These enzymes are also distinct from the *recA* gene product, which catalyses the ATP-dependent proteolytic cleavage of the λ repressor during prophage induction^{34,35}, but does not hydrolyse casein or globin to acid-soluble material (data not shown). Regnier and Thang²⁵ partially purified a casein-hydrolysing activity, named protease A, which was eluted from DEAE-cellulose at the same ionic strength as peak II. This peak contains three enzymes that differ in their isoelectric points, sensitivities to inhibitors and substrate specificities (Fig. 3). Thus, protease A is a mixture of proteases, rather than a single enzyme.

The marked stimulation (4–40-fold) of peak IV protease by ATP can account for the energy requirement for protein breakdown *in vivo*^{1,8,9}. In crude extracts, a much smaller stimulation of proteolysis by ATP (60–200%) was observed¹⁰, probably because of the five additional globin-degrading enzymes active in the absence of ATP. Previous studies^{8,30} indicate that the initial endoproteolytic steps in protein breakdown require ATP, and it is therefore likely that these steps are catalysed by the ATP-stimulated protease in peak IV. Recently, this enzyme has been shown to be the product of the *lon* gene (also called *CapR* and *deg*)^{32,33,36,37}, and *lon* mutants have a reduced capacity to degrade abnormal proteins^{36,37}.

When cells are disrupted by gentler methods than those used here, a large amount of ATP-stimulated proteolytic activity is associated with the cell membrane³⁰. It is not clear whether the ATP-stimulated activities in the soluble and membrane fractions are different enzymes or whether the soluble activity is

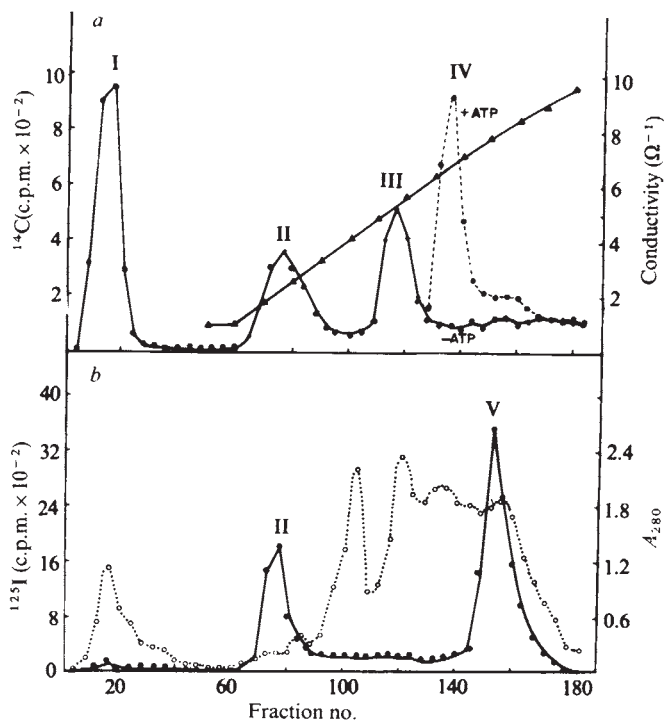


Fig. 1 DEAE-cellulose chromatography of proteolytic enzymes from *E. coli* K-12. In *a*, fractions were assayed for proteolytic activity against ^{14}C -globin in the absence (●—●) or presence (○—○) of 3 mM ATP. In *b*, proteolytic activity against ^{125}I -insulin (○—○) was assayed. Also shown in *a* is the conductivity (▲—▲) and in *b*, the A_{280} (○—○). In all peaks except peak IV, the hydrolysis of ^{14}C -globin and ^{125}I -insulin was not stimulated by ATP (for clarity, these data were not shown). The globin-degrading peaks also hydrolysed ^3H -casein (data not shown). Cell-free extracts were prepared from 50 g of frozen *E. coli* K-12 cells (Grain Processing Co.). After thawing at 4 °C, the cells were suspended in 100 ml of 50 mM Tris-HCl, pH 7.8, 10 mM MgCl_2 and 200 mM KCl, and disrupted in a French press at 14,000 p.s.i. The homogenate was centrifuged at 30,000g for 30 min, and the supernatant centrifuged again at 130,000g for 3 h. The supernatant was dialysed overnight against 10 mM Tris-HCl, pH 7.8, containing 5 mM MgCl_2 . The dialysed extract was adsorbed to a DEAE-cellulose column (2.5 × 27 cm) equilibrated with 10 mM Tris-HCl, pH 7.8, containing 5 mM MgCl_2 . The column was washed with the same buffer until the A_{280} of the eluate was <0.05. The adsorbed proteins were then eluted with 1,600 ml of a linear NaCl gradient (0–0.25 M NaCl). The flow rate was 100 ml h^{-1} and 12-ml fractions were collected. Proteolytic activity was estimated by following the degradation of ^{14}C -globin (2.4×10^6 c.p.m. per mg), ^3H -casein (1×10^7 c.p.m. per mg) or ^{125}I -insulin (125–140 mCi per mg, Cambridge Nuclear and Radiopharmaceutical Corporation) to products soluble in 10% trichloroacetic acid. ^{14}C -globin was prepared by methylating crystalline beef haemoglobin (Sigma) with ^{14}C -formaldehyde (40–60 mCi mmol^{-1} , NEN) and bovine α -casein with ^3H -formaldehyde (40–60 mCi mmol^{-1}) as described previously⁴⁸. To increase its proteolytic susceptibility, haem was extracted from the haemoglobin using methyl-ethylketone⁴⁹. All the assays were carried out in 50 mM Tris-HCl, pH 7.8, 10 mM MgCl_2 , in the presence or absence of 3 mM ATP. When assayed with globin or casein as a substrate, the incubation mixture contained 100–200 μl of enzyme preparation and 4–5 μg of ^{14}C -globin or 25 μg of ^3H -casein in a final volume of 0.5 ml. Assays using insulin as a substrate contained 12.5 μg insulin (Lilly), a trace amount of ^{125}I -insulin (12–15,000 c.p.m.) and the enzyme preparation in a final volume of 0.5 ml. The incubations were performed for 2 h at 30 °C when globin or casein was the substrate, and for 1 h at 37 °C when insulin was the substrate. After incubation, 40 μl of bovine serum albumin (30 mg ml^{-1}) was added as a carrier and 60 μl of 100% trichloroacetic acid to precipitate the proteins. The assay tubes were kept on ice for 30 min, and after centrifugation, 0.4 ml of acid-soluble products from ^{14}C -globin or ^3H -casein hydrolysis were counted in 4 ml of Liquescent (National Diagnostics). The products of ^{125}I -insulin hydrolysis are counted in an autogamma spectrometer. The protein was determined by the method of Lowry *et al.*⁵⁰ using crystalline bovine serum albumin as standard.

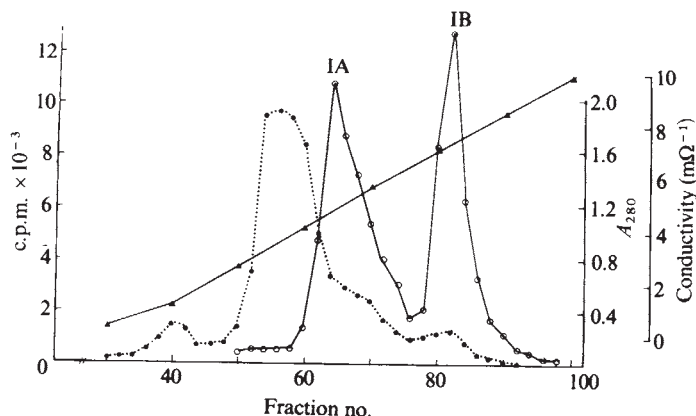


Fig. 2 CM-cellulose chromatography of proteases in peak I. Hydrolysis of ^3H -casein (○—○), A_{280} (●—●) and conductivity (▲—▲) are shown. The pooled fractions from peak I of the DEAE-cellulose column (Fig. 1) were concentrated and dialysed against 10 mM sodium acetate buffer, pH 5.6. After removing the precipitated material by centrifugation, the proteins were loaded onto a CM-cellulose column (1.5 × 12 cm), equilibrated with 10 mM sodium acetate buffer, pH 5.6. The adsorbed proteins were eluted with 400 ml of 0–0.3 M linear NaCl gradient. The flow rate was 35 ml h^{-1} and 4-ml fractions were collected.

Table 1 Proteolytic activities from *Escherichia coli*

Peaks	Stimulation by ATP	Inhibitors	Suggested nomenclature
Globin and casein-degrading enzymes			
IA	—	DFP	Do
IB	—	DFP	Re
IIB	—	DFP	Mi
IIC	—	DFP	Fa
III	—	DFP	So
IV	+	DFP	La
Insulin-degrading enzymes			
IIA	—	EDTA, <i>o</i> -phenanthroline	Pi (periplasmic)
V	—	<i>o</i> -Phenanthroline	Ci (cytoplasmic)

released from a membrane-associated form during cell breakage. Some of the other soluble enzymes described here may also be derived from the inner or outer membranes during cell disruption.

A major task for future research will be to clarify which of these enzymes are involved in the degradation of abnormal proteins and in the breakdown of normal cell constituents in starving cells. These proteases may also have critical roles in other physiological processes, such as the processing of secretory or membrane proteins³⁸⁻⁴¹, utilization of exogenous peptides²⁸, phage morphogenesis^{28,42}, breakdown of colicins⁴³⁻⁴⁵ and inactivation of regulatory proteins^{34,42,46}. One important clue to the physiological roles of these enzymes is their subcellular distributions. Elsewhere we shall demonstrate that one of the insulin-hydrolysing activities is found in the periplasm (IIA) and one in the cytoplasm (V). One globin-degrading activity (IIB) is localized in the periplasm, one (IB) seems to be distributed equally between periplasm and cytoplasm, and the others (IA, IIC, III, IV) are cytoplasmic. Presumably, these latter enzymes are involved in the catabolism of intracellular proteins.

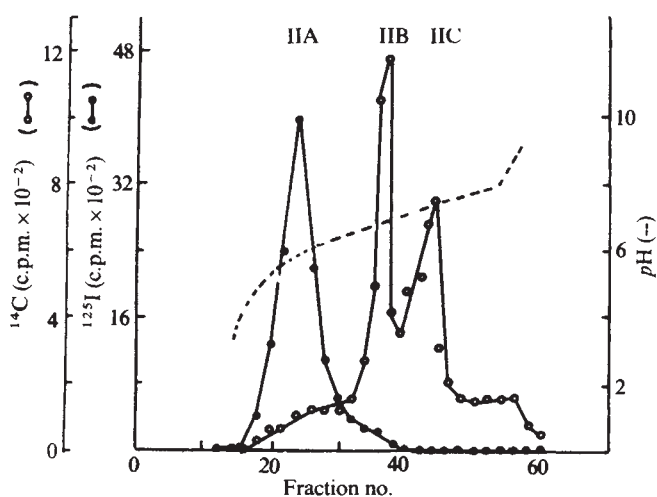


Fig. 3 Isoelectric focusing of peak II. The peak II fractions from DEAE-cellulose column (see Fig. 1) were combined and concentrated by precipitating the proteins by adding solid ammonium sulphate to 95% saturation. After centrifugation at 31,000g for 30 min, the pellet was dissolved, dialysed against 1% glycine overnight and applied to the isoelectric focusing column. Isoelectric focusing was performed using a LKB 110-ml column in a sucrose gradient with 1% solution of Ampholine in the pH range 5–8. Electrofocusing was conducted for 22 h at 4°C with a constant voltage of 1,600 V. Fractions (2 ml) were collected from the bottom. The pH of the fractions was measured at 4°C (---) and 0.2-ml aliquots were assayed for activity against ¹²⁵I-insulin (●) and ¹⁴C-globin (○). The peaks of IIA, IIB and IIC have isoelectric points (pI) of 5.75, 6.75 and 7.36, respectively.

The discovery of seven new enzymes necessitates the choice of a useful nomenclature. Unfortunately, the lack of precise information about their physiological functions or chemical specificities precludes informative designations. Therefore, we have decided to introduce a novel nomenclature for these enzymes. As the two metalloenzymes that degrade insulin have distinct compartmentalizations in the cell⁴⁷, we suggest the name Pi for the periplasmic insulin-degrading enzymes (IIA) and Ci for the cytoplasmic insulin-degrading enzyme (V) (Table 1). Such a scheme, based on subcellular distribution, is not useful for the enzymes that degrade globin and casein. We would like to introduce an easily remembered nomenclature for these six enzymes: proteases Do, Re, Mi, Fa, So and La in their order of elution from DEAE-cellulose and CM-cellulose or isoelectric focusing (Table 1). This nomenclature also permits an easily remembered designation for their genetic loci. When more is known about the properties or roles of these enzymes, a more descriptive nomenclature will be possible.

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Requirement of *nifV* gene for production of wild-type nitrogenase enzyme in *Klebsiella pneumoniae*

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Klebsiella pneumoniae nitrogenase is a complex enzyme consisting of two component proteins, the tetrameric MoFe protein (Kp1) and the dimeric Fe protein (Kp2)¹. Together they catalyse the ATP-dependent reduction of N₂ and of several triple-bonded substrates such as C₂H₂, CN⁻, N₃⁻ and CH₃NC (refs 2, 3). In the absence of substrate, protons are reduced to H₂ and all substrates compete with protons for reduction. Carbon monoxide inhibits all reductions except H₂ evolution⁴. Seventeen genes are required for the synthesis of nitrogenase and expression of its activity in *K. pneumoniae*⁵⁻⁸, some of which are believed to be involved in processing of the nitrogenase proteins during synthesis^{9,10}. Here we describe studies of the nitrogenase of bacteria mutated at one of these genes—*nifV*. These mutants produce nitrogenase which does not reduce N₂ *in vivo*, although it does *in vitro* in conditions of high electron flux. We show that the defect is in the Kp1 component, and suggest that the *nifV* gene product modifies the Kp1 component so that N₂ reduction will occur at the lower electron fluxes present *in vivo*, and that this might have been involved in the possible evolutionary development of the nitrogenase from a cyanide-detoxifying enzyme.

nifV mutants were originally isolated by their inability to grow anaerobically (anaerobic conditions are required because oxygen represses nitrogenase production) on nitrogen-free medium, but all *nifV* mutants were shown to give a leaky phenotype when assayed for C₂H₂ reduction^{5,6}, a test normally accepted as indicative of N₂-reducing ability. Indeed, some *nifV* :: Tn7 mutants gave 50–100% of wild-type C₂H₂-reducing levels even though such mutations should completely inactivate the *nifV* gene product in most cases⁶.

To test the true nitrogen-fixing ability of *nifV* strains, derepressed cultures were incubated under an atmosphere of ¹⁵N₂ and after 11 h cell pellets were collected for ¹⁵N analysis (Table 1). The two *nifV* :: Tn7 mutants gave no significant incorporation of ¹⁵N into cell material whereas these mutants significantly reduced C₂H₂, indicating that derepression had occurred (Table 1). Failure to incorporate ¹⁵N₂ was not due to defective assimilation of fixed nitrogen^{11,12}, because both mutants utilized histidine, arginine, tryptophan and nitrate as sole nitrogen sources aerobically and nitrate and arginine anaerobically. If N₂ cannot act as a substrate for the enzyme the presence of N₂ might not affect C₂H₂ reduction. Figure 1 shows the effect of N₂ on *in vivo* C₂H₂ reduction by whole cells of a *nifV* mutant (UNF1613) and a wild-type control (UNF3001). C₂H₂ reduction by wild-type cells was noticeably inhibited by 0.1 atm and 1 atm N₂ whereas C₂H₂ reduction by the mutant was not. UNF3001 and UNF1613 had a *K_m* for C₂H₂ of 4.2 and 4.6 atm, respectively. We conclude that *nifV* insertion mutants are unable to reduce N₂ *in vivo*.

This inability of *nifV* mutants to reduce N₂ could be due to an excess of Kp1 over Kp2, resulting in a situation favouring C₂H₂ reduction^{13,14}. Crude extracts of *nifV* and wild-type strains were thus tested for the relative levels of the two nitrogenase proteins by rocket immunoelectrophoresis using purified proteins as standards. These showed that UNF3001, UNF1610 and UNF1613 crude extracts had an *in vivo* Kp2:Kp1 molar ratio of 6.6, 5.2 and 4.7, respectively. These results are not significantly different and confirm the conclusion¹⁵ that there is an effective excess of Kp2 *in vivo*. However, this assumes that all cross-reacting material is catalytically active. Unfortunately, direct activity measurements on the individual nitrogenase proteins in crude extracts is complicated by their interdependence for

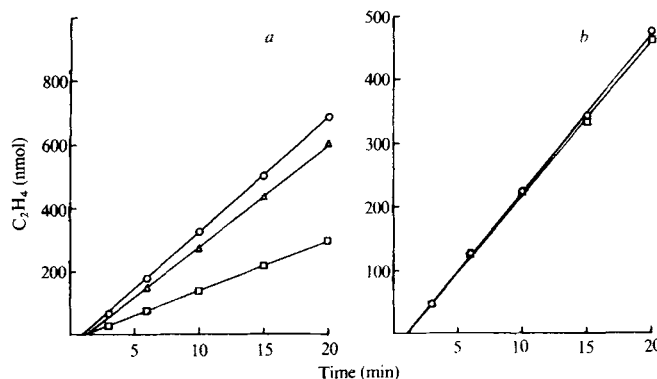


Fig. 1 Time course for *in vivo* C₂H₂ reduction by wild-type (UNF3001) (a) and *nifV* mutant (UNF1613) (b) cells at 0.0074 atm C₂H₂. Cultures were grown in NFDm + arginine (1 g l⁻¹) for 20 h at 30 °C with constant N₂ bubbling. They were collected by centrifugation under argon and resuspended in cold argon-bubbled NFDm + chloramphenicol (0.1 g l⁻¹) and kept on ice under argon. Samples (5 ml) were taken by syringe into 30-ml flasks flushed with either argon or N₂ and incubated for 10 min at 30 °C before addition of 0.25 ml C₂H₂ to start the reaction. Headspace samples (0.5 ml) were taken at intervals and analysed by gas chromatography. Assays under 0.1 atm N₂ were set up by adding 3.0 ml N₂ to the 29-ml headspace. ○, 1 atm Ar; △, 0.1 atm N₂ in Ar (wild type only); □, 1 atm N₂.

substrate reduction. An indirect estimate of the extent to which Kp1 is saturated with Kp2 in crude extracts can be measured by observing the increase in C₂H₂-reducing activity on addition of excess purified Kp2. Figure 2a shows the effect of adding a >10-fold molar excess of purified Kp2 protein to crude extracts of a wild-type and *nifV* strain. Kp2 addition stimulated C₂H₂-reducing activity to approximately the same extent in both crude extracts. Thus, an unfavourable Kp2:Kp1 ratio is unlikely to account for the inability of *nifV* cells to fix N₂ *in vivo*.

Figure 2b shows hydrogen evolution by crude extracts under various atmospheres and the effect of added Kp2. Control assays omitting either ATP or dithionite showed that <10% of H₂ evolved in assays with added Kp2 was attributable to other hydrogenase activity. Nitrogen failed to compete with protons in assays with mutant extracts even with the addition of a sevenfold molar excess of Kp2 (maximum 6% inhibition of H₂ evolution) whereas significant competition (63% inhibition) was seen in assays of the wild-type extract even without added Kp2. Thus, the *nifV* crude extract is apparently defective in reducing N₂ as a substrate, although it reduced CN⁻ and CH₃NC (data not shown). CO alone or CO plus C₂H₂ did not significantly affect H₂ evolution in the wild-type extract (Fig. 2b). (The combination of CO plus C₂H₂ inhibits reversible hydrogenase without affecting H₂ evolution by nitrogenase¹⁶.) However, hydrogen evolution by *nifV* crude extracts was inhibited 50% in the presence of 3%

Table 1 Incorporation of ¹⁵N₂ by cultures of *K. pneumoniae nifV* mutants

Strain	Genotype	Specific activity of C ₂ H ₂ reduction (% of wild-type)	Atom % ¹⁵ N enrichment
UNF2036	<i>nifΔ</i>	<1	<0.4
UNF3001	<i>nif</i> ⁺ (wild-type)	100	21
UNF1610	<i>nifV</i> 2249::Tn7	61	0.7
UNF1613	<i>nifV</i> 2253::Tn7	81	0.5

Strains were grown under a stream of N₂ for 8 h at 30 °C in nitrogen-free Davis and Mingcoli medium (NFDm)⁶ arginine (1 g l⁻¹) to permit derepression. Cells were collected by centrifugation, resuspended in fresh Argon-bubbled NFDm + arginine (1 g l⁻¹) and shaken for 11 h at 30 °C under 63% N₂ (70% enriched for ¹⁵N₂) in argon. Cell pellets were collected by centrifugation and digested for ¹⁵N analysis by mass spectroscopy. C₂H₂ reduction was measured before incubation under ¹⁵N₂ as in Fig. 1 except that 2.0 ml of culture was used.

Table 2 Effect of varying the molar ratio of purified Kp2 and *nifV* Kp1 on substrate reduction

Source of Kp1	Kp2:Kp1 molar ratio	Specific activity of substrate reduction (nmol per min per mg Kp1)				% Inhibition of H ₂ evolution by N ₂	Electron flow under N ₂ Electron flow under Ar ×100
		H ₂ under argon	H ₂ under N ₂	NH ₄ ⁺ production	C ₂ H ₂ reduction		
UNF1613 <i>nifV</i> ::Tn7	1:1	182	155	17.8	152	15	100
UNF1613 <i>nifV</i> ::Tn7	2::1	399	340	73.5	320	15	113
UNF1613 <i>nifV</i> ::Tn7	4::1	763	503	157	620	34	97
UNF1613 <i>nifV</i> ::Tn7	7::1	1,022	717	259	843	30	108
UNF1613 <i>nifV</i> ::Tn7	10::1	1,242	842	318	1,044	32	106
UNF1613 <i>nifV</i> ::Tn7	25::1	1,532	938	370	1,262	39	97
M5al wild type	1::1	249	94.4	113	247	62	102
M5al wild type	10::1	1,527	471	725	1,127	69	102

Assays were done with 0.22 μM MoFe protein either under 1 atm Ar, 0.13 atm C₂H₂ in Ar or 1 atm N₂ at 30 °C, and were linear with time. H₂, C₂H₂ and NH₄⁺ were determined as described previously²¹. Specific activities in nmol C₂H₄ reduced per min per mg protein in optimal conditions were *nifV* Kp1, 1,262; wild-type Kp1, 1,240; wild-type Kp2, 1,150. Component ratios were calculated assuming a maximum specific activity for C₂H₂ reduction of 1,800 nmol per min per mg for both Kp1 and Kp2.

CO or CO plus C₂H₂. Thus ATP- and reductant-dependent H₂ evolution by *nifV* nitrogenase is inhibited by carbon monoxide, in contrast to wild-type enzyme.

The failure of added Kp2 to stimulate N₂ reduction might be due to inhibition by defective Kp2 in the mutant crude extract, a situation analogous to certain heterologous combinations of nitrogenase components from different organisms¹⁷⁻²⁰. Following purification of Kp1 and Kp2 from UNF1613 by conventional procedures^{19,21,22}, Kp2 from the *nifV* mutant showed wild-type properties with respect to N₂ reduction and CO insensitivity of H₂ evolution when complemented by wild-type Kp1 protein. The *nifV* Kp1 showed CO inhibition of H₂ evolution when complemented with wild-type Kp2 (Fig. 2c). These mutants therefore have a defective MoFe protein.

Table 2 shows the effect of the Kp2:*nifV* Kp1 molar ratio on substrate reduction. The rate of substrate reduction increased with increasing Kp2:Kp1 ratio. The rate of NH₄⁺ production by the mutant Kp1 increased with ratio but was less than half the wild-type rate at a 10:1 ratio. Even at a 25:1 ratio only 39% of the electron flow was utilized for NH₄⁺ production, compared with 62% for a 1:1 ratio with wild-type Kp1. However, the rate of electron flow through both mutant and wild-type enzyme was the same under either argon or nitrogen (Table 2 and ref. 23). Clearly, N₂ reduction by *nifV* Kp1 only competes effectively with hydrogen evolution at non-physiological molar ratios of the two proteins, whereas C₂H₂ reduction seems to be normal. The

discrepancy between the lack of N₂ reduction *in vivo* and in crude extracts compared with the measurable rates with purified enzyme may reflect the presence of other proteins in the former which affect the rate of nitrogen reduction.

Recent *in vitro* studies with purified nitrogenase components have shown that the distribution of electrons between competing substrates depends on the rate of electron flux through the MoFe protein and that this is influenced by the ATP concentration and the Fe:MoFe protein ratio^{13,14,24}. The electron flux increases with increasing Fe protein:MoFe protein ratio^{24,25} and whereas low electron fluxes favour proton reduction, progressively higher fluxes favour C₂H₂ and N₂ reduction in that order²⁵. As *nifV* Kp1 does not respond normally to Kp2 addition, what is the function of the *nifV* protein? We conclude that it processes the MoFe protein, and that this modification renders H₂ evolution insensitive to CO inhibition. Furthermore, the *nifV* product seems to modify the catalytic ability of Kp1 so that N₂ reduction will occur at a lowered electron flux. It has been suggested that nitrogenase was originally a cyanide detoxifying enzyme²⁶. We suggest that the *nifV* modification might be a later evolutionary development which converted the original enzyme into an efficient nitrogenase. Finally, like other workers^{14,17}, we stress the need for caution when using the C₂H₂-reduction test as a measure of nitrogen fixation. The study of the *nifV* MoFe protein may provide useful insights into the structure of the MoFe protein of nitrogenase and its mechanism of substrate reduction.

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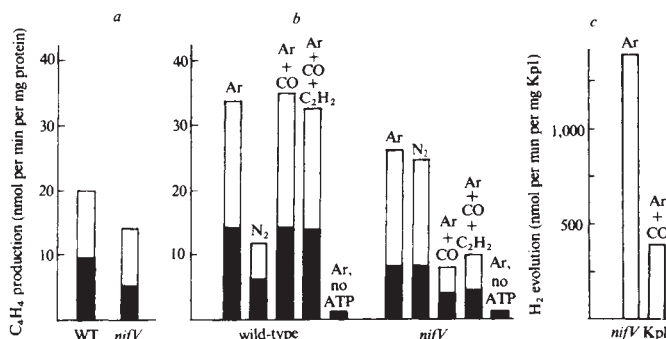


Fig. 2 a, C₂H₂ reduction by UNF3001 (wild type, WT) and UNF1613 (*nifV*) crude extracts with (unshaded) and without (shaded) added Kp2; b, H₂ evolution by crude extracts under argon; N₂; Ar+3% CO; Ar+CO+14% C₂H₂. 20-min assays were done as in ref. 21. Cells for crude extract preparation were grown for 17-18 h in 20 l Ar-bubbled NFDH + NH₄Cl (0.24 g l⁻¹). Sodium aspartate was added (50 mg l⁻¹) and the pH adjusted to 7.2. After 5 h derepression, cells were collected by centrifugation anaerobically, resuspended in 30-45 ml 50 mM HEPES pH 8.7, 0.65 mM dithiothreitol and 2 mM Na₂S₂O₄, crushed at 12,000 Pa in a French pressure cell and cleared by centrifugation at 38,000g for 45 min. The supernatant was frozen in liquid N₂ and stored at -196 °C. Assays²¹ contained 1.03 mg (UNF3001) and 1.54 mg (UNF1613) protein and were linear with added protein. Kp2 (specific activity 900 nmol C₂H₂ per min per mg) was added at 0.151 mg per assay where appropriate. c, H₂ evolution by purified *nifV* Kp1 and a 10-fold molar excess of wild-type Kp2 under argon with and without 5.8 matm carbon monoxide.

BOOK REVIEWS

Synaptic patterns of the mind

H.B. Barlow

THIS book is based on a Work Session of the Neurosciences Research Program that was held in May 1978, but although it is thus based on papers given at a conference three years ago it is very much alive and worth reading by those interested in theoretical approaches to the nervous system. The editors have evidently made a determined effort to present the material as a coherent whole in a readable form, and to a large extent they have succeeded. They divide the material up into three levels: the nature of the computations that are needed for the sensory and motor tasks we perform, the algorithms by which the computations are achieved, and the biological mechanisms which perform them. These are dealt with in reverse order, neural mechanisms first and the nature of the computation last; the recognition of the three levels provides a convincing framework for describing selected aspects of the enormously complicated processes that underlie our perceptions and control our movements. The book not only has a unified viewpoint but also an original feature here visible, I think, for the first time. This is the attempt to provide a theoretical background, together with a shorthand graphical representation, for the non-linear interactions on which the crucial logical operations of the nervous system depend. However let us first consider the review chapters, which are still useful even though not fully up to date.

The first section has four chapters on how the nervous system performs its computational algorithms. The first two are useful general reviews by T. Lamb and D. Baylor on the biophysics of transduction in photoreceptors and the integration and transmission of this information through the retina. These look upon signal generation and transmission as linear processes, but the next two chapters by T. Poggio and V. Torre are the ones in which a biophysical mechanism for the non-linear interactions that must underlie pattern selective mechanisms are proposed, and I shall return to them for they form the book's kernel of originality.

The next section has seven chapters at the level of "algorithms and interactions" in neural systems. It contains up-dating reviews on various aspects of the visual system by Victor, Poggio, Reichardt, Wilson, Julesz, Hubel and Cowan. As the editors seem to admit, it is not quite right to apply the term "algorithm" to what is here described. An algorithm surely has a clearly defined purpose, but we are almost completely ignorant of the purposes of

Theoretical Approaches in Neurobiology. Edited by W.E. Reichardt and T. Poggio. Pp.252. ISBN 0-262-18100-2. (MIT Press: 1981.) \$27, £14.

non-linear interactions in the cat's retina, cooperative processes in texture perception or the columnar organization of the visual cortex. All the same one needs a level of description intermediate between biophysics and artificial intelligence or cognitive modelling. Anyone who has tried to understand someone else's computer program, or even one of his own, will recognize the dazed feeling that accompanies the question "What in the world does this page of nonsense accomplish?", and that is the feeling one has when reading much of this middle section of the book. Of course something is accomplished, but the interpretive keys are in many cases unknown, or at any rate not divulged.

The level of the final section is that of describing the nature of computations. Two of them deal with visual control of flight in flies (Reichardt and Poggio), and of limb pointing in monkeys. The other two are concerned with human perception. Ullman deals at a theoretical level with the remarkable human capacity to interpret the relative motions of a few isolated spots of light in terms of the coherent motion of a single rigid object. Marr's chapter gives a brief summary of his approach to vision, and it would form a very useful introduction to his work for someone who has not read his lengthier papers. It is clear that this book as a whole owes a great deal to Marr's recognition of the different levels of description that are required in order to understand visual perception.

Tomaso Poggio, as well as editing the book, is author or co-author of at least 25 per cent of the material in it, and in these sections an important new approach is set out. As Boole realized when he wrote the "Laws of Thought" more than 100 years ago, higher mental processes require logical operations that are essentially non-linear and more like multiplications than addition or subtraction. He was of course thinking of mental processes at a verbal level, but the lesson must also apply for the pre-verbal processes that underlie pattern selectivity and motor control, and it is the mechanism and organization of these processes at the level of the synaptic interactions between cells that Poggio and Torre attack in the biophysical section of this book. The intracellular potential of a particular cell has customarily been regarded as summing the positive and

negative influences of all the neurones that make excitatory and inhibitory synaptic connections with it. Poggio and Torre point out that synapses situated close to each other on one of a cell's dendrites will mutually influence each other, and the effects produced at a distant point will not simply represent the sum of the two. For instance, if one synapse is inhibitory and acts by increasing conductance to an ion whose concentration ratio is close to that corresponding to the cell's resting potential, then its activation will contribute very little potential change at a distant point in the cell. Nevertheless it may have a very powerful effect in stabilizing the potential at that point in the cell when a neighbouring excitatory synapse becomes active. This shunting effect can be very powerful, but will only be local. They propose that this may be the mechanism whereby directional selectivity is achieved in retinal ganglion cells, and it certainly fits two of the main requirements deduced from physiological analysis, namely that the interactions responsible are between local sub-units of the receptive field of a retinal ganglion cell, and that they are inhibitory in nature. The logical operation is thus equivalent to "and not", or one input vetoing another, rather than to the conjunctive "and".

The development of this idea is given in some detail, made more digestible by placing much of the mathematics in an appendix, as is also done in the rest of the book. Unfortunately, though the ideas are important for a large number of people, it is not very easy to follow. The authors have sought clarity in exactitude, whereas a certain amount of over-simplification might have been more effective. They also invent an interesting graphical notation which has symbolic representations for conductance changes as synaptic inputs, for the currents thereby produced and the potential changes resulting from the filter-like action of decremental electrotonic conduction, and most important, for the multiplicative effect of a conductance change on a voltage. If this notation is developed, and is adopted, one may be able to "read" a neural circuit in the same way that one can transistor circuits. It may even be possible to relate such a graphical form of neural circuit to the synaptic anatomy visible by electron microscopy, for many important quantities determining non-linear interaction are dependent upon anatomical variables such as proximity and dendritic diameter.

Neurophysiology is mainly a simple

science, and I am not sure that Poggio has expressed his ideas in a simple enough form for us to understand. But this part of the book is certainly a novel attack on a most important problem, and one leaves the book as a whole in a rather more optimistic mood than is usually the case after a dose of brain theory. Instead of feeling that the subject matter is incoherent and totally unapproachable, one feels that, within the framework set out, the brain may, ultimately, become comprehensible. □

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Not quite Allard

C.N. Law

The Theory of Plant Breeding. By O. Mayo. Pp.293. ISBN 0-19-854536-3. (Clarendon/Oxford University Press: 1981.) £30, \$89.

PLANT breeding has long required an up-to-date and authoritative textbook. It is now over 20 years since what many regarded as a good book on the subject, *Principles of Plant Breeding* by R.W. Allard (Wiley, 1960), first appeared. Since that time numerous attempts have been made to write a worthy replacement but with limited success. This book on the theory of plant breeding, by O. Mayo, is likely to receive a similar assessment. This is a pity, since the book has merit and touches on much that needs to be said about plant breeding and related sciences.

The book has 17 chapters, of which seven of the longest describe methods of genetic analysis, the properties of genetic systems in inbreeding and outbreeding crops, the response of such systems to environmental variables and different selection pressures. These are then followed by several short chapters on induced mutation, disease resistance, somatic cell genetics and, as if as an afterthought, further cytogenetical manipulations. The final chapters discuss the conservation of germplasm and aspects of plant breeding strategy.

A number of errors occur, mainly of omission. Thus, the author is rather cursory in his treatment of the covariance-variance analysis of diallel crosses, and is apparently not aware that the creators of this analysis have been at pains to point out its weaknesses in detecting certain types of gene action. Likewise, the treatment of the genetical causes of heterosis leaves much to be desired, particularly since the author appears to labour under the misconception that heterosis is a phenomenon still awaiting a genetical explanation.

Far too frequently important methods and issues are mentioned *en passant*, when

Entomology — and how the West was won

Philip S. Corbet

From Arsenic to DDT: A History of Entomology in Western Canada. By Paul W. Riegert. Pp.357. ISBN 0-8020-5499-4. (University of Toronto: 1981.) \$30. To be published in the UK on September 24, £18.

THE history of Canadian entomology has received close and expert scrutiny before. Compared with two previous accounts — by R. Glen (*Can. Ent.* **88**, 290–371; 1956) and G.J. Spencer (*Can. Ent.* **96**, 33–59; 1963) — Professor Riegert's attractively presented book is narrower in scope but much more detailed. It traces the development of entomology (mainly applied entomology) in the four western provinces of Canada, from the arrival of the first European colonists until just after the Second World War, when synthetic organic chemical insecticides were starting to transform the practice of pest control. (The history of forest entomology is omitted because Riegert discovered that such a document had recently been prepared.) As it stands, this book provides a wealth of detail on subjects as varied as the daunting mosquito attacks recorded by traders in the early seventeenth century, instructions for making and applying "Criddle Mixture" (a horse-manure-based poisoned bait for grasshoppers) and the progressive evolution and enforcement of interprovincial quarantine regulations.

The five main parts of the book are devoted to encounters between European colonists and insects; the first professional entomologists; the insects of British Columbia — mainly mosquitoes and orchard pests; the insects of the prairies — including the wheat-stem sawfly, grasshoppers, cutworms and wireworms; and specialized topics such as medical and veterinary pests, stored-products insects, and the teaching of entomology in universities.

A brief, admirably succinct, epilogue reviews the main personalities and events which attended the growth of applied entomology in western Canada from the 1880s to the 1950s — "from arsenic to DDT" — and ends by listing some of the lessons to be learnt from this richly documented set of case studies.

more detailed and penetrating accounts are demanded. This is a disappointing feature which probably arises from the stated objectives of the book, which are to give an account of plant breeding theory but not to provide extensive descriptions of proofs or methods. However, one questions whether this is a wise decision when such an important aspect as breeding for disease resistance receives only three pages of text. The impression emerges that the author has much to contribute in writing about the

The author's style tends to be somewhat florid for my taste, and his inclusion of diverse anecdotal and factual material at times impairs the fluency of the text, although this seems a modest price to pay for the exhaustive background information he provides. A map would have been a useful adjunct to the excellent contemporary photographs.

This book will certainly appeal to those wishing to learn, or to be reminded of, the circumstances and individuals that have helped Canadian entomology to become so vigorous and effective; and, for the sake of the discipline's continuing health, one may wish that such readers will include those who decide the resources to be allocated to entomology by future Canadian governments! As a contribution to the existing literature on the history of entomology, Riegert's book has undoubted value in several other respects. Using a canvas of manageable size, he delineates the changing pattern of agricultural and veterinary entomology in a developed, though recently settled, country which is prone to severe pest problems. The extremely detailed description of these problems — including contemporary recipes for their mitigation — and the frank but sensitive description of the men and institutions concerned with pest management, may attract the interest of social historians as well as applied entomologists. One may hope that those destined to practise and administer applied entomology in Canada and elsewhere properly appreciate the realities of large-scale pest suppression when synthetic organic chemical pesticides are not available. People who read this book will surely gain such an appreciation; and in doing so they will come to share the author's high regard for the immensely able and resourceful pioneer entomologists who successfully tackled some of the most intense insect attacks ever to be endured by human beings.

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theory of plant breeding; but either he has been overly constrained by limitations on the size of the book or he has been perhaps too unambitious in his objectives. He should be encouraged to build upon the firmer foundations of this book as well as to include views about the practice of plant breeding in any future edition. □

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Illiterate lamp men and other problems in geodesy

William M. Kaula

Geodesy, 4th Edn. By G. Bomford. Pp.855. ISBN 0-19-851946-X. (Oxford University Press: 1980.) £49, \$139.

In 1952 I was reassigned from a combat engineer battalion in the Fulda Gap to the Ohio State University in order to study geodesy. Upon arriving in Columbus that June, I met Professor W. A. Heiskanen, who said: "I'm going to Finland tomorrow for the summer. Here, study this book". "This book" was the first edition of Bomford's *Geodesy*, just published. So that summer I spent many hours between classes under the trees (now gone) on the east bank of the Olentangy, gleaning geodesy from *Geodesy*.

Geodesy is a compendium, rather than a textbook or a treatise. It is the principal geodetic reference book in English. The main parts of this new edition are: horizontal control (196 pp.), vertical control (57 pp.), physical geodesy (118 pp.), artificial satellites (139 pp.), and appendixes and bibliography (186 pp.).

Virtues of the book and its author include comprehensiveness within geodesy, a strong geometrical intuition, keen insights about instrumentation and observations, a sensitivity to measuring errors — and good writing. Bomford has much better command of language than most scientists and engineers. His discussions of such topics as the effects of atmospheric refraction on theodolite or spirit-level measurements are a pleasure to read, being syntheses of practical experience, an analytical attitude and fluency of expression. His Indian past is manifest in passages such as "When supplies and transport are easy and with lamp men who can find their own way about, the programme presents no special problems, but in uninhabited country, or with illiterate lamp men, it calls for very careful thought . . .". Bomford's careful selection of tangential topics, practical (e.g. star catalogues) or scientific (e.g. geophysical implications of gravity anomalies) is also evident.

However, he is less at ease with the abstract and the mathematical. His treatments of potential fields and orbital dynamics are somewhat perfunctory, and these topics are made unnecessarily obscure by quoting complicated formulae, rather than discussing underlying principles. A saving grace is the frequent referencing; anyone trying to use *Geodesy* as a text (as I did) must resort to looking up the references to understand whence came

many of the prickly-looking equations. Oddly incongruous with the quoted formulae in the main text are most of the appendixes, which include rather elementary explanations of matrix algebra, theory of errors, vectors and so on. Some of this matter is outmoded: for example, eight pages on the solution of matrix equations make no mention of square-root techniques, while four pages on harmonic analysis reference only books written before 1930, and do not mention fast Fourier transform methods. Such archaisms may unduly deter those who have had modern engineering educations from using the book.

The revisions between the 1971 and 1980 editions are major. The type appears to have been completely reset and the size of the book has risen by 17 per cent. This increase is almost entirely explicable by the greater space accorded to artificial satellite methods, which has not only more than tripled but has also been rearranged with respect to the other material. However, going through the two editions in parallel reveals many other detailed changes, such as increases in the space devoted to crustal movements, time standards, MSL-geoid difference and gyro-theodolites which are balanced by decreases in that allocated to map projections, mechanical chronographs, astrolabes etc. In the earlier parts, particularly, this compressive zeal was paragraph by paragraph. A loss of flavour sometimes results. Thus, what was in the 1952 edition:

Such an apparatus was used in India in 1880 [150], p.35, and another was used with the prismatic astrolabe in 1927-9 [151], but neither was entirely satisfactory. Possibly the principle was sound, but the mechanism imperfect. Recently R. Woolley (of the Commonwealth Observatory, Canberra) has made an apparatus on similar lines, which is reported to be satisfactory in the observatory, although possibly not easily portable. Full reports have not yet been published.

has now shrunk to: "Such an apparatus has been constructed from time to time, but no great use of the method has been reported". Unfortunately, this drive to prune and mend apparently flagged by the time the appendixes were reached.

Bomford's experience and perception in geodetic survey is particularly applicable to current problems of using past geodetic measurements to infer crustal movements. The relevant cautionary remarks appear in both the sections on control and those on crustal movements: for example "The differences cannot lightly be ascribed to crustal movements", and "Levelling has sometimes been less good than it looks". Several likely systematic errors are discussed, but the bite of specific examples is lacking, perhaps because the Survey of India was always stricter in its practices

than was the US Coast and Geodetic Survey.

I must report that I found no errors, other than a few misprints. The defects are much more ones of selection and exclusion. In addition to those mentioned above, an oddity is the omission of the determination of longitudinal variations in the gravity field from satellite orbit perturbations, which is both conceptually simpler and more fruitful of geodetic results than the resonant effects which are included.

Nonetheless, if you have a geodetic problem, look it up in Bomford. If he doesn't give you the answer, he'll tell you where else to look. □

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Material attraction

P. J. Grundy

Ferromagnetic Materials: A Handbook on the Properties of Magnetically Ordered Substances. Vols 1 and 2. Edited by E.P. Wohlfarth. Vol.1 pp.634, ISBN 0-444-85311-1. Vol.2 pp.600, ISBN 0-444-85312-X. (North-Holland: 1980.) Each volume Dfl.210, \$102.50.

MANY textbooks and technical handbooks lay claim to the title of the definitive text. However, in order to prove its superiority over all comers, a book, like all championship contenders, must have all the necessary qualities. If all the promised material — a further two volumes are planned — is provided and published within a reasonable time, this work should, indeed, prove to be a champion.

The handbook is designed to cover as wide a range of material properties as possible. Its restrictive title of *Ferromagnetic Materials* is quickly extended by the subtitle to take account of the other varieties of magnetic ordering, and the subjects covered by the first two volumes are varied indeed. The contributing authors have been well chosen, are "experts" in their specialist fields and most are household names in magnetism.

Understandably, there is no obvious overall plan to the layout of the chapters, except that the first is by the editor and reflects his deep interest and involvement with work on the trio of ferromagnetic transition metals. The second chapter of the first volume, by Mydosh and Nieuwenhuys on dilute transition metal alloys, deals authoritatively with the complex and fascinating range of effects arising from the concentration changes and differing magnetic interactions of the

The first of a new series, *Current Reviews in Biomedicine*, has been published by Elsevier/North-Holland. The paperback *Towards Understanding Receptors*, edited by J.W. Lamble, is a well-organized collection of 33 reviews largely taken from *Trends in Pharmacological Sciences*, and costs £7, \$25.

magnetic impurity in these spin glass systems. This first volume also contains contributions on rare earth metals and alloys and compounds by Legvold, Buschow and Clark. Each of these authorities has worked on these fundamentally important, numerous (Buschow lists details and references to at least 1,000 binary and ternary compounds) and potentially useful materials since the 1960s. The journey through the periodic table is completed by Trzebiatowski's contribution on actinide elements and compounds. Even for many people active in magnetism, these materials are somewhat enigmatic. However, their properties are clearly dealt with and no text could claim to be comprehensive without their inclusion. As might be expected, the chapter on amorphous magnetic materials prepared by rapid quenching from the melt is an especially authoritative and lucid account; Luborsky and co-workers have been pace-makers in the study of the magnetic properties and preparation methods of these alloys, especially those suitable for the application and production of commercially useful material.

The second volume is dominated by contributions from the research and development laboratories of large industrial corporations. This is an important and welcome feature for several reasons. One is that it brings home to the reader, especially in the chapters on bubble domain materials by Eschenfelder, where charts in which interrelated material properties and parameters can be cross-checked are given, the special approach to research which is concerned with designing and using materials in a real application. Another is that articles, such as those by Chin and Wernick on soft magnetic materials and Bate on magnetic recording materials, are extremely comprehensive and are accompanied by exhaustive reference lists — presumably the product of excellent information retrieval systems. On the other hand, the smaller corpus of knowledge on other materials, and perhaps a more selective approach, is evident in some of the other contributions. This also has its advantages in that it can be said that part of the author's responsibility is to guide the reader and select the important contributions to his subject.

The four articles by Gilleo, Slick, Nicolas and Eschenfelder on the magnetic ferrites and garnets cover the ground admirably. The final chapter by Charles and Popplewell is, not surprisingly, the only contribution on materials which are effectively liquids. "Ferrofluids" present a challenge in thought as to what uses can be made of magnetizable colloidal suspensions. Several applications have already been addressed but one severe problem seems to be the rapid departure from liquid behaviour accompanying only modest increases in concentrations and magnetization.

There is no doubt that these volumes

should be available in all research and development laboratories concerned with magnetic materials and material properties in general. Their value as sourcebooks to both graduate students and seasoned researchers is obvious, and their appearance will help to promote the important messages that magnetic materials display a bewildering variety of properties and that the applications of magnetism

permeate the whole of modern society by providing electric power, communications and information storage. I am sure the international magnetism community awaits with anticipation the volumes yet to appear. □

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Mathematical stimulus for biologists

J.D. Murray

The Geometry of Biological Time. By Arthur T. Winfree. Pp.530. ISBN 0-387-09373-7/3-540-09373. (Springer-Verlag: 1980.) DM 59.50, \$32.80.

GENUINE interdisciplinary research can often produce spectacular and exciting results. Winfree, a biologist, knows a lot of mathematics and this book reflects his appreciation of interesting problems in both fields. Amongst other things he gives some excellent case histories of what interdisciplinary endeavour can achieve. There is no doubt that the development of models and model mechanisms as an aid in elucidating biological phenomena with a view to determining basic principles is a flourishing, useful and fascinating area of research. (The interdisciplinary mathematics-biology fields are now recovering from the ravages caused by catastrophe theory.) In many countries the generous support from research funding organizations reflects this growth area.

This book is a major contribution to theoretical biology and will become, I am sure, an essential reference book for those, theoreticians as well as experimentalists, interested in modelling and understanding periodic behaviour in biology. From a pedagogical point of view it does not require much detailed knowledge of mathematics as such, except for selected chapters, but it does require a mathematical appreciation and understanding which most bioscientists do not have. However, those with firmity of purpose, who persevere with most of the more instructive first nine chapters, will reap rich rewards. Those with less time or weaker will can usefully go to those parts of the second half of the book which deal with more specific phenomena. Many of the ideas and concepts described are illustrated with relevant, and often ingenious experiments.

Winfree first discusses periodic behaviour in general, phases, phase singularities and phase resetting. The latter is shown to be an important concept of relevance to experimental observation. The effect of interactions between oscillators is then examined. In particular, where they are spatially distributed they can result in a

variety of unexpected wave phenomena. One of Winfree's aims is to try to give a picture of observed events and natural phenomena which are not dependent on specific models. Generally he uses a geometric conceptual approach.

The second half of the book deals mainly with specific phenomena and experiments which exemplify some of the ideas developed earlier, but can be read, more or less, independently from the first half. Here Winfree discusses several major topics of current biological research and among other things gives his personal views on them. For example, such topics as the Belousov-Zhabotinskii reaction (emphasizing the wave phenomenon aspect), slime mould aggregation, circadian rhythms in insect eclosion, the female cycle and the cell mitotic cycle are surveyed and discussed. Researchers in each of these areas, and others covered, will no doubt feel that the views expressed do not give the whole picture of current thinking (and how could they in a single book?) or present a biased picture, and so on. This indeed so, but whatever their views, it will be difficult to ignore many of Winfree's ideas with impunity.

This book exhibits a remarkably wide spectrum of biomedical problems of current research interest. With Winfree's talent in presenting succinctly the essential interesting features of a phenomenon, it is accessible to most mathematicians, physical scientists, and with somewhat more effort, bioscientists who believe in the use of models in biology (and few can now admit to a contrary view). Parts of it can be usefully given to both undergraduates and graduate students. I strongly recommend it for those seriously or casually interested in modelling and understanding biological oscillators and their role in nature: the book is full of unanswered questions and research ideas. As an introduction for the uninitiated, and a stimulant for the cognoscenti, it is excellent, lucidly and anecdotally written, and has a well-vetted bibliography. □

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